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INSTITUTT FOR PATOLOGISK ANATOMI UNIVERSITETET I OSLO
(HEAD OLAV TORGERSEN M D)

NEPHROTIC SYNDROME WITH CYST LIKE DILATATIONS OF RENAL TUBULES

Report of 2 Cases in Siblings in Early Infancy

By

AKSEL A ONGRE

Received 8 vii 60

The present paper is an account of three siblings who clinically suffered from the same renal disorder probably representing a nephrotic syndrome They died at the ages of about four six and two weeks respectively In two of the cases the kidneys were examined with routine histological methods combined with microdissection of nephrons

Although nephrotic syndrome in early infancy has repeatedly been reported in recent years microdissection of nephrons has apparently been performed in only few cases *Giles et al* (1) have published three cases in two of which the patients were siblings

REPORT OF CASES

The two oldest children of this siblingship are alive and healthy six and four years of age summer 1958 The following three infants of these parents died however in the first weeks of life

Case 1

Clinical course This patient a boy was born March 1 1955 three weeks before term The pregnancy was uneventful When he was 18 days old he
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The clinical course was dominated by *pronounced edema severe proteinuria* and *repeated vomitings* which were not projectile The body temperature during the
° C He died on March 29 1955 Necropsy

of congenital renal disease The final

Case 2

Clinical course The patient a girl was born June 11 1957 at her home The pregnancy and delivery were probably normal Neither physician nor midwife was

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SUPPLEMENTA

INSTITUTT FOR PATOLOGISK ANATOMI UNIVERSITETET I OSLO
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The case was c ns lered to be an instance of congen tal renal d sease The final diagn sis was nephropathy

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Figs 1 5 (Case 2)

Fig 1 Kidney Hematoxyline and eosin Note tubular dilatations being most prominent in the deeper part of the cortex *Fig 2* X 125 Proximal convoluted tubule Note the irregular outline with "pouching" or diverticule like dilatations *Fig 3* X 125 Distal tubules joining collecting tubule At the point of junction one of the distal tubules is atrophic, and proximal to this a dilatation is seen The rest of the tubule is broken off *Fig 4* X 128 Two distal tubules are joining collecting tubule Note dilatations proximal to junctions The rest of the tubules is broken off

Fig 5 X 192 A dilatation in the course of a tubule is demonstrated

present at birth and no information concerning birth weight and measures is available. A midwife arriving half an hour later found mother and child in good condition.

The patient appeared normal until the age of two weeks. At that time she presumably presented generalized edema. A physician consulted said that this child also probably suffered from renal disease.

Nothing further is known about her development until she was 40 days old when her abdomen suddenly became increasingly distended and vomitings occurred.

She was admitted to hospital July 22 1957 with a diagnosis of intestinal invagination. Physical examination revealed a mongoloid appearance, a distended and tense abdomen, slightly decreased muscular tonus, good turgor of the extremities but decreased turgor of the abdominal skin. The body temperature was 37.7° C. Urinalysis showed positive albumin and benzidin reactions. The amount of urine was insufficient for further examination. Blood hemoglobin was 113 per cent. X-ray examination of the abdomen showed ascites but no signs of intestinal invagination. Abdominal paracentesis was performed and 550 ml of a whitish fluid was removed. Specific gravity of the fluid was 1.006 and reactions for protein and fatty acids gave positive results.

The next day, July 23, the child was transferred to Oslo University Hospital. On examination late in the evening the patient was moribund showing no spontaneous activity, had almost inaudible heart sounds and a pulse so feeble that it could not be counted. Muscular tonus was decreased and abdominal and tendon reflexes could not be elicited. The pupils were about one millimetre in diameter and nonreactive to light. [illegible]

[illegible]

about 100 ml of a [illegible] The fluid contained 245 mg/100 ml of total lipids and traces of cholesterol.

Other findings included slightly increased amounts of a clear translucent fluid in the pericardial and pleural cavities, hypostatic changes of the lungs and a few slightly enlarged mesenterial lymph nodes. The spleen weighed 13 gms and was of firm consistence.

Microscopic examination. In the kidneys many of the glomeruli had hyalinized areas of varying size and location within the capillary tufts. These areas showed positive PAS reaction. Individual glomeruli showed slight epithelial proliferation [illegible] signs of adhesions were [illegible] signs of cell prolifera[illegible] lesions showed no zone [illegible] cortex.

Pathological tubular changes were conspicuous, most severe in the deeper part of the cortex, less prominent in the subcapsular area and the medulla. Several tubuli showed varying degrees of cyst-like dilatation while others [illegible] atrophic. The [illegible] from well [illegible] tubular ep[illegible] amounts [illegible] substance which was the case also in a few glomeruli. Double refractile lipids were not found.

The contents of the dilated tubules were a homogeneous eosinophilic substance

1

with a strongly positive PAS reaction positive Millon's test, and it also contained small amounts of sudanophilic substance Hemosiderin (Turnbull) and hemoglobin (Lepehne) reactions were negative

The greater part of the medulla appeared normal There was a slight increase of connective tissue throughout the kidneys The intrarenal vessels and the renal pelves were normal

In the central parts of *the brain* especially in sections from the thalamo hypo thalamic region the blood vessels showed fibrous thickenings and were surrounded by a definite lymphocyte infiltration In addition neuronophagia and microglial increase were present

The lungs showed considerable atelectasis mainly of subpleural localization In some of the alveoli there were edema and macrophages Scattered interstitial hemorrhages and dilated capillaries and blood vessels were also found

The liver had normal structure There was scanty vacuolization of the liver cells as also dilatation and congestion of the central veins and adjacent central parts of the sinusoids A brown pigment was observed in some of the liver cells and sinusoids in the latter probably situated in the Kupffer cells The pigment gave a negative Turnbull reaction

The spleen showed signs of congestion and some brown pigment in a few macrophages

In sections from the small and large intestine located in the adventitia and adjacent part of the mesentery, were found scattered small infiltrations of a few lymphocytes granulocytes and macrophages The lymph vessels appeared normal though a few were probably slightly dilated The mesenteric lymph nodes had a slightly dilated subcapsular sinus with a few sinus macrophages

Microdissection of nephrons was performed according to methods used by several authors (2 3 4 5) During dissection the tubules had a tendency to break in atrophic areas and small amounts of connective tissue hampered the dissection to some extent Thus, although only fragments could be mounted successfully valuable information was gained during dissection of the material

Most of the nephrons looked abnormal Especially in the subcapsular cortex they were either short (as compared with nephrons from a normal infant of about the same age) with few convolutions and no distinct loop of Henle or with a descending and ascending limb without any differentiation The glomeruli seemed normal

The proximal tubules showed few and inconstant changes They often had a slightly irregular outline with small local dilatations

The severest and most constant lesions were in the distal parts of the nephrons These changes were more prominent in the juxtamedullary cortex though found also in other areas In the course of the distal tubules single or multiple balloonings' or cyst-like dilatations were found the parts of the tubule adjacent to the dilatations were atrophic It was at these points that the tubules broke during the mounting The dilatations were of varying appearance some spherical some spindle shaped and some convoluted The balloonings were frequently found close to the junction between the distal convoluted tubules and the collecting tubules The collecting tubules appeared normal

Some of the features described are demonstrated see figures

Case 3

(Information about this patient and a kidney specimen was kindly put to our disposal by *Professor F Waaler M D* at The Gade Institute University of Bergen)

s born slightly before term

dark yell w At 8 days of

rsity of Bergen with anasarca The total serum proteins were 2 3 gms per 100 ml

Autopsy was performed at The Gade *Institute University of Bergen* (no 8 59 The organs especially the elastic tissue were deeply jaundiced A generalized edema was present The liver was dark and firm The gallbladder contained clear fluid and the common duct was patent The hepatic ducts were not examined The intestinal contents showed only trace *of bilirubin* (0 8 mg%) Microscopy of the liver showed multiple bile thrombi in the bile capillaries

The kidneys were large and white Microscopically the cortex was narrowed with

fibrosis in many glomeruli others showed adhesions to the capsula and in several there was epithelial proliferation with crescent formation The tubuli showed a remarkable dilatation with thinning of the epithelium particularly in the deep layer of the cortex

Additional serological investigation were performed in serum from the mother with extract of formaline fixed kidney tissue from the child as antigen in compliment fixation reaction precipitation test and indirect haemoagglutination reaction

As a result of the investigations at The Gade Institute it was concluded that it was a case of congenital glomerulonephritis presenting itself clinically as a "nephrotic syndrome" The tubular changes were considered to be secondary to a primary glomerular lesion No antibodies were demonstrated in the mother s serum against the child s kidney The retention type jaundice was considered as an incidental finding and remained unexplained (atresia of the hepatic duct?, pressure from edema? allergy?)

At our institute we have had the opportunity to study a kidney specimen Our histological and microdissection examinations revealed similar lesions in case 2 and 3 except that proliferative changes were more prominent in the latter In this case the tubular changes also seemed most prominent in the juxtamedullary cortex the terminal part of the distal tubules being most affected

Genetic Aspects

An investigation regarding consanguinity between the patients' parents was made The investigation was carried out by Humangenetisk Laboratorium University of Oslo, and the results were kindly put at our disposal by Dr *Jan Mohr* No consanguinity between the parents, the grandparents or the parents of the maternal grandfather was demonstrated

COMMENTS

In these cases clinical and post-mortem findings direct attention to renal pathology The proteinuria and edema in the first case, the proteinuria edema and ascitic fluid in the second, and the anasarca and hypoproteinemia in the third case favour the conception that all these siblings presented a nephrotic syndrome

Evidently the cases do not fulfil the commonly accepted criteria albuminuric proteinuria, hypoalbuminemia, edema, and hypercholesterolemia But because of the patients early death there is no available information on all of these points The ascitic fluid containing lipids, in case 2 could not be explained by local anatomical findings In a case of nephrotic syndrome in a neonatal premature infant described by *Fiben Kleinerman & Cline* (7) a milky fluid with a specific gravity of 1 006 and proteins of 30 mg per 100 c c was removed by abdominal *paracentesis*

Considering the renal changes we have not come to a final conclusion as to the nature of the lesions The glomerular lesions with hyalinizations and slight proliferative changes necessitate a discussion of glomerulonephritis On the other hand the tubular lesions are a peculiar feature favouring the concept of a primary tubular disease

In other reports on nephrotic syndrome in early infancy the renal lesions have differed *Kunstadter & Rosenblum* (12) have reported a

with a strongly positive PAS reaction, positive Millon's test and it also contained small amounts of sudanophilic substance. Hemosiderin (Turnbull) and hemoglobin (Lepehne) reactions were negative.

The greater part of the medulla appeared normal. There was a slight increase of connective tissue throughout the kidneys. The intrarenal vessels and the renal pelves were normal.

In the central parts of *the brain*, especially in sections from the thalamo-hypothalamic region, the blood vessels showed fibrous thickenings and were surrounded by a definite lymphocyte infiltration. In addition neuronophagia and microglial increase were present.

The lungs showed considerable atelectasis mainly of subpleural localization. In some of the alveoli there were edema and macrophages. Scattered interstitial hemorrhages and dilated capillaries and blood vessels were also found.

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Some of the features described are demonstrated (see figures).

Case 3

(Information about this patient and a kidney specimen was kindly put to our disposal by *Professor F. Waaler, M.D.*, at The Gade Institute, University of Bergen.)

This boy was the 5th and the last of the children. He was born slightly before term and weighed 2500 gms at birth. The urine was permanently dark yellow. At 8 days of age the mother noticed that he was edematous.

The child was admitted to the Children's Hospital, University of Bergen, with anasarca. The total serum proteins were 2.3 gms per 100 ml.

Autopsy was performed at The Gade Institute, University of Bergen (no. 8/59). The organs, especially the elastic tissue, were deeply jaundiced. A generalized edema was present. The liver was dark and firm. The gallbladder contained clear fluid and the common duct was patent. The hepatic ducts were not examined. The intestinal contents showed only trace of bilirubin (0.8 mg%). Microscopy of the liver showed multiple bile thrombi in the bile capillaries.

The kidneys were large and white. Microscopically the cortex was narrowed with

pathway of renal injury is a known feature, seen for instance in multiple myeloma (15) It has also been observed by *Hanssen* (6) following intraperitoneal injection of bacitracin in mice After 72 hours protein casts occurred in many distal convoluted tubules and to some extent also in the loop of Henle and the distal parts of the proximal convoluted tubules the latter also being significantly dilated After seven days he found atrophic and degenerated tubules embedded in a loose connective tissue appearing among areas of histologically normal tubules Experiments revealed that there was a decrease in the normal tubular reabsorption of proteins and that ferrocyanide did not pass the mechanical obstruction made by the protein casts

A similar mechanism might be present in our cases The protein casts might constitute a mechanical obstruction in the course of the tubules At these points the tubules become dilated, and the nephrons undergo atrophic and degenerative changes proximally and distally In this way a vicious circle is started within the kidneys Thus the glomerular changes seen in the light microscope as well as the interstitial connective tissue proliferation might be of a secondary character

The causes of protein cast formation are many such as the concentration of urea electrolytes hydrogen ions and proteins and alterations in the nature of proteins In our cases one also must think of an inborn tubular enzyme defect

In accordance with the changes in the distal part of many nephrons a disturbance at the points of union with the collecting tubules must be taken into account Thus the possibility of cystic kidneys is within range the findings supporting the non-union theory However, there were to many normal junctions and there is no remarkable similarity with the microdissection findings in a case of cystic kidneys reported by *Bialestock* (16)

In case 2 the patient had hypertelorism right-sided epicanthus, and long and slender fingers and toes Could this point to an embryopathy?

It is a peculiar feature in the present material that the most advanced lesions are found in the juxtamedullary cortex In the phylogenetically younger subcapsular cortex many of the nephrons were of the premature type with few or no convolutions lacking the loop of Henle, or having a descending and ascending limb without any differentiation It is a problem why the phylogenetically oldest part of the cortex is most affected During the first period of nephronogenesis *an injurious* agent may have acted upon the embryo and led to a dysfunction in the juxtamedullary nephrons On the other hand conditions for cast formation may not be present in the subcapsular premature nephrons

SUMMARY

A description is given of three siblings who probably presented a nephrotic syndrome shortly after birth and died at the ages of four, six,

case of neonatal glomerulonephritis and nephrotic syndrome in a 1,320-gms prematurely born infant. A case of congenital glomerulonephritis with nephrotic syndrome in a 2500-gms prematurely born infant has been published by *Frischknecht, Zollinger & Keiser* (8). In the case reported by *Eiben et al.* (7), the birth weight was 2110 gms and the pathological findings compatible with the diagnosis of lipoid nephrosis. *Hallman, Hjelt & Ahvenainen* (9) have given an account of eight cases. Most of the infants were born before term with birth weights less than 2500 gms. The renal changes were compatible with the diagnosis of chronic proliferative nephritis in three cases, in two of which the disease had run the longest course. However, the glomerular changes varied and in the two infants who had lived the shortest time, only slight changes could be detected in the mesangial cells, together with thickening of the basal membranes of the glomerular capillaries. Thus "the glomerular changes increased almost consistently with an increase in age at death". In all the cases, including those in which the glomerular lesions were slight, the tubules had become filled with albumin masses. In addition there were distinct signs of fatty degeneration.

Giles et al. (1) have microdissected the kidneys in three cases of nephrotic syndrome at this age. In two of their cases the infants were siblings whose parents and paternal grandparents were first cousins. The renal lesions in our cases have much in common with those described by *Giles et al.*, considering both histological and microdissection findings. Although tubular changes differed somewhat with regard to the degree, appearance, and the localization of lesions, similar glomerular changes seem to have been made. These are considered secondary to tubular lesions, by *Giles et al.* Similar glomerular changes can also be seen in cases of unilateral hydronephrosis and in cases of sudden death in infancy (13, 14).

The electron microscopical studies by *Hjelt, Stjernvall & Hallman* (10) on congenital nephrotic syndrome, and by *Farquhar, Vernier & Good* (11) on familial nephrosis revealed changes in the glomerular epithelium, basement membrane, and slight changes also in the endothelium. These findings point to the glomerular apparatus as the seat of lesions in nephrotic syndrome. But *Hjelt et al.* also found a laminated structure of the tubular basement membranes. They discuss the possibility of basement membrane changes occurring concurrently in glomeruli and tubuli.

Turning to the present cases, the occurrence of three probably identical cases in one siblingship points to a single etiology and pathogenesis. The early start of clinical symptoms and signs, and the advanced anatomical lesions found at an early death are compatible with an origin in utero.

For some unknown reason there *probably exists a disorder with protein cast formation*, most severe in the distal tubules and in the juxtamedullary cortex. Tubular obstruction of intratubular origin is a

FROM THE PATHOLOGICAL DEPARTMENT OF SABBATSBERG HOSPITAL (HEAD PROF N RINGERTZ) INSTITUTE OF RADIOPATHOLOGY (HEAD PROF L SANTESSON) AND ROENTGEN DIAGNOSTIC DEPARTMENT D (HEAD DOCENT A LINDBOM) STOCKHOLM

MULTIPLE CAPILLARY HEMANGIOMAS OF THE BONES OF THE FOOT

By

S O Lidholm A Lindbom and H J Spjut[1]

Received 13 vii 60

Hemangiomas primary in bone that give clinical signs and symptoms may still be classed as uncommon lesions Most of the reported hemangiomas have been histologically classified as the cavernous type Capillary hemangiomas of bone are rare

Sherman in 1941 collected five capillary hemangiomas of bone including a case of her own These included cases reported by *Bucy & Capp 1930 Geschickter & Maseritz* 1938 and by *Abbott*, 1941 *Geschickter & Copeland* 1949 described four capillary hemangiomas three of the humerus and one of the ulna The latter case had been reported earlier by *Bucy & Capp* One of the first capillary hemangiomas of bone reported occurred in a clavicle and was described by *Phemister* 1929 (The journal was not available to us) A capillary hemangioma of the fibula in a 64 year old man was reported by *Hadders & Rinsma*, 1955 Multiple capillary hemangiomas of the skull have been described by *Handousa* 1947 The supporting photomicrographs of Handousa's case and that reported by Abott are somewhat less than convincing for capillar [illegible]
Rockemer [illegible]
and skull a [illegible]
diagnose th [illegible] capillary hemangioma but only as hemangioma For this reason and that the photomicrographs are not convincing for capillary hemangioma the cases are not considered as such in this paper

The purpose of this paper is to report a patient with multiple proved capillary hemangiomas of the bones of the foot In addition the long follow up period (24 yr) and interval radiographs may give a clue concerning the life history of capillary hemangiomas of bone To our knowledge this would be the eleventh case of capillary hemangioma of bone described if the two probable cases in the skull are included

[1] Address Division of Surgical Pathology Washington University Medical School St Louis Mo USA

and two weeks respectively The kidneys were studied by histological methods combined with microdissection, with additional serological studies in case 3

Glomerular changes mostly consisted of partial hyalinizations and slight proliferative changes Tubular changes were most severe in the juxtamedullary cortex, but seen also in other areas The proximal tubules showed few changes, but in the distal tubules pronounced cyst like dilatations with intraluminal hyaline casts were found The majority of the neprons in the subcapsular cortex were of the premature type

Comments are made with reference to a few cases resembling the present ones Some considerations of the problem of pathogenesis in the present cases are also mentioned

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Fig 1

CASE PRESENTATION

1936 because
had pain and
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On physical examination the left ankle was slightly swollen Light pressure over the medial malleolus produced tenderness Pain could be produced by twisting the foot

Initial roentgenograms of the left foot showed multilocular cystic structures in the calcane
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foot sl
Bloc
were within normal limits

On Nov 3 1936 an exploration of the neck for parathyroid adenomas and a curettage of the lesion of the talus were carried out Adenomas were not found in the neck The talus biopsy contained a small fragment of tissue compatible with capillary hemangioma

On Nov 18 1936 the tumor of the calcaneus was curetted Two "peasized" pink blue tumors were also removed from the soft tissues just posterior to the medial malleolus Histologically the tissues were interpreted as an angiomatous tumor (Fig 4)

From 1936 to 1944 the patient noted slight pain in the left foot and a small slowly enlarging mass at the previous operative site The foot was injured again in 1944

TABLE 1

Summary of Published Cases

Author	Age and sex		Major symptoms	Location	Treatment	Follow up
Abbott 1941	3 yr	♀	—	Skull	Local resection	8 mo well
Bucy & Capp 1930	20 yr	♂	Tumor after injury of the right wrist	Lower end of ulna	Local resection	5 yr well
Geschickter & Copeland 1949	—		—	3 cases in humerus	—	—
Geschickter & Maseritz 1938	8 yr	♀	Tumor after injury of the left arm	Left humerus	Curettage and X ray treatment	5 yr well
Haldeks & Binsma 195?	64 yr	♂	*Tenderness of the left lower leg*	*Left fibula*	Local resection	—
Hand & Bey 1947	36 yr	♂	Exophthalmus and headache	Skull bones	Local resection	—
Thiemister 192?				*Clavicle*	—	—
Sherman 1944	1 mo	♀	Swollen left knee	Lower end of femur	Curettage	2 yr well
Wilhelm, Lindbom & Spjut 1960	20 yr	♂	Pain and tenderness of the left foot after injury	Calcaneus, cuboid, talus and fifth metatarsal of the right foot	Curettage and X ray treatment	24 yr well

Fig 1

Radiograph of the left foot made in 1944 shows radiolucent cystic appearing lesions of the head of the fifth metatarsal, cuboid and calcaneus The lesion in the talus is obscured by superimposed bones Posteriorly bone reaction to the surgical procedures of 1936 is noted

CASE PRESENTATION

Case 460 Bone Tumor Registry of Radiopatologiska Institutionen

The patient was a 20 years old man admitted to the hospital in May 1936 because of injury to the left foot six months previously Since the injury he had pain and slight swelling of the foot near the ankle and Achilles tendon Pain was more pronounced with exercise Prior to the accident there had been no signs or symptoms related to the foot

On physical examination the left ankle was slightly swollen Light pressure over the medial malleolus produced tenderness Pain could be produced by twisting the foot

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Lidholm Lindbom & Spjut 1960	20 yr ♂	Pain and tenderness of the left foot after injury	Calcaneus cuboid talus and fifth metatarsal of the right foot	Curettage and X ray treatment	24 yr well



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The patient was a 20 years old man admitted to the hospital in May 1936 because of injury to the left foot six months previously Since the injury he had pain and slight swelling of the foot near the ankle and Achilles tendon Pain was more pronounced with exercise Prior to the accident there had been no signs or symptoms related to the foot

On physical examination the left ankle was slightly swollen Light pressure over the medial malleolus produced tenderness Pain could be produced by twisting the foot

Initial roentgenograms of the left foot showed multilocular cystic structures in the c[illegible]
were [illegible]
foot [illegible]
[illegible]
were [illegible] limits

On Nov 3 1936 an exploration of the neck for parathyroid adenomas and a curettage of the lesion of the talus were carried out Adenomas were not found in the neck The talus biopsy contained a small fragment of tissue compatible with capillary hemangioma

On Nov 18 1936 the tumor of the calcaneus was curetted Two "peasized" pink blue tumors were also removed from the soft tissues just posterior to the medial malleolus Histologically the tissues were interpreted as an angiomatous tumor (Fig 4)

From 1936 to 1944 the patient noted slight pain in the left foot and a small slowly enlarging mass at the previous operative site The foot was injured again in 1944

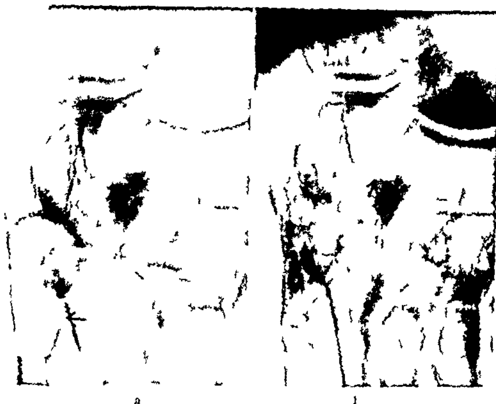


Fig 2a
The lesions of the fifth metatarsal and cuboid as seen in 1944

Fig 2b
Roentgenogram in 1958 showing sclerosis of the head of the fifth metatarsal and no alteration of the lesion in the cuboid bone



Fig 3a and 3b
Radiographs from 1944 and 1958 respectively show little or no change in the lesions in the calcaneus and talus

Fig 4

Second biopsy in nov 1936 from the calcaneus showing a characteristic capillary hemangioma The first biopsy was similar (van Gieson × 490)

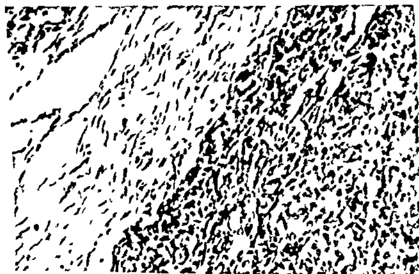
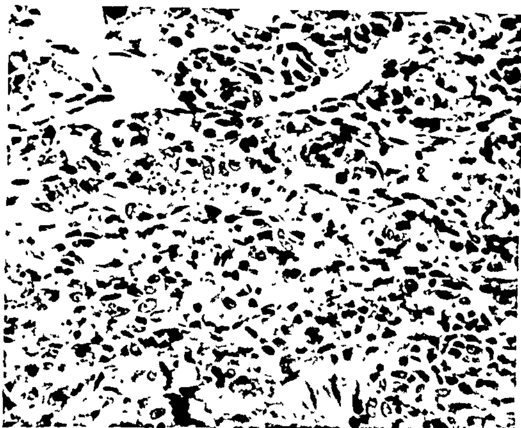


Fig 5

Hemangiomatous nodule from the abductor hallucis muscle excised in 1945 Upper left showing parts of the striated muscle (H E × 340)



I ig 6
Capillary hemangioma curetted from the base of the fifth metatarsal in 1945
(H E × 490)

In March 1945 because of the recurrent tumor he was admitted to a hospital and an operative procedure was done A "doves eggsized" bloody tumor was removed from the talus The abductor hallucis muscle was excised because of the presence of several small blue red nodules within the muscle A friable bloody mass was also curetted from the base of the fifth metatarsal (Fig 6) The n dules in the muscle were not continuous with those in the bone Pathologically the lesi ns were inter preted as multiple angiomatous tumors suspicious for malignancy

Irradiation therapy was given in 1945 over a 4 week period to the left foot 2 000 r to a medial field and 2 000 r to a lateral field (Calculated tumor d se 2 400 r)

The p   of Radiumhemmet at yearly
interval   nplaints of pain he has re
mained   in 1960

Radiographic Findings

Roentgenograms of the left foot in 1936 showed cystic lesions of four bones the calcaneus cuboid talus and head of the fifth metatarsal The lesions were radio lucent and were quite well outlined by faintly sclerotic margins These films were not available for review Films taken in 1944 dem nstrated the cystic lesions and the effects of the operations d ne in 1936 (Fig 1) There was irregular sclerosis of the talus and in the superior portion of the calcaneus Judging from the 1936 de scription there had been no changes in the other lesi ns over the eight year period Between 1944 and 1958 the lesions of the calcaneus and cub id remained wholly unchanged The lesion of the cuboid had never been biopsied *The lesions of the talus and fifth metatarsal showed irregular b ney scler sis at the 1958 examinati n* During the observation peri d there was no evident progressi n of the lesi ns (Figs 2a 2b 3a 3b)



Fig 7

Photomicrograph of the tumor in the talus showing intact bone and areas of capillary hemangioma This tumor, removed in 1945 is similar to that in Fig 4 (H E × 200)

Pathological Findings

Since the lesions of our case were removed by curettage, little can be said concerning the gross features except that the tissue was bloody Of interest are the multiple pink blue and red blue nodules in the soft tissues and abductor hallicus brevis muscle These nodules also proved to be capillary hemangiomas (Fig 5) These lesions were not attached to bone and not continuous with the hemangiomas in the bones

Histologically the hemangiomas from the talus calcaneus cuboid and soft tissues were characteristic of the capillary type The tumors were richly vascular and cellular Blood vessels larger than capillaries were noted in a few areas of each of the biopsies The capillary component was overwhelmingly predominant In the soft tissue hemangioma the lesions were lobulated as well as occurring as separate foci Review of all of the histologic sections did not reveal microscopic alterations of a malignant vascular tumor

In bone [illegible]
seemed isc[illegible]

[illegible] were noted in the hemangiomas after previous biopsy procedures Mitotic figures were very rare Overall each of the lesions was similar to a capillary hemangioma that might be found in the skin

DISCUSSION

Multiple hemangiomas of bone have been reported before (*Ackermann & Hart* 1942, *Paden & Matz* 1955) In the sense of multiplicity our case is not unusual The remarkable features of our case are the multiple capillary hemangiomas of bone and soft tissue as well as 24

years follow-up period with interval radiographs available for study. From the standpoint of evolution the various lesions showed no tendency to progress; bony sclerosis followed the surgical procedures. The role of the irradiation is diffucult to assess in the face of the surgical procedures and the fact that the lesion in the cuboid bone remained radiographically unchanged. It is within the realm of possibility that *spontaneous regression of capillary hemangiomas* could occur as is the case in many of those of the skin (*Modlin*, 1955). There is a difference, however, in the skin regression occurs at an early age whereas the majority of capillary hemangiomas of bone in which the age is known have been discovered in adolescent or adult patients.

From our material we are unable to add information as to the origin of hemangiomas, whether hamartomatous or neoplastic.

SUMMARY

A case of a 20 years old man with multiple capillary hemangiomas of the bones and soft tissues of the foot has been presented.

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FROM THE HISTOLOGIC DEPARTMENT OF THE KAROLINSKA INSTITUTET, STOCKHOLM 60 HEAD PROFESSOR G HÄGGQVIST

THE INFLUENCE OF THYROXIN ON BLOOD LYMPHOCYTES

A Quantitative Study of the Mitochondrial Contents of Blood Lymphocytes in Guinea Pigs

By

U LENNSTRÖM and B LARSSON

Received 20 vii 60

The administration of thyroxin subcutaneously in young guinea pigs gives rise to a lymphatic hyperplasia (see *Gyllensten* 1953) provided that a general cachexia has not established itself Such hyperplasia is much more pronounced in the lymph nodes than in the spleen and thymus In these hyperplastic nodes the histologic picture shows a conspicuous proliferation of cells in the marrow, and the differential cell counts disclose a relative increase of the plasma cells and of all the transitional cell types ranging from reticular to plasma ones (*Ernstrom & Gyllensten* 1959) However, the hyperplasia cannot account entirely for the added weight of the lymph nodes There is in all likelihood also an increased thickness of the cortical layers the lymphocyte being the dominating cell type Furthermore a qualitative study *indicated* a larger number of secondary follicles after prolonged thy[illegible]

s

[illegible] *normal* development of lymphoid tissue and of induced lymphatic hyperplasia (cp *Wiseman* 1931, *Andreasen* 1943) The average age of the blood lymphocytes is stated to decrease in fair correlation to *the development* of lymphatic hyperplasia The age of the lymphocyte has been judged by its size but this is not, in *Wiseman's* opinion (1931) a reliable measure Wiseman instead suggested the cytoplasmic contents of *RNA* or the contents of mitochondria as the best indicators of lymphocytic *age* The latter has been used by several investigators and has proved to be in fair correlation to the prolifera tion and hyperplasia of the *lymphoid* tissue under physiological and pathological conditions

Dur[illegible]

after [illegible]

drin [illegible] cyte in the rabbit (*Osogoe, Chang, Awaya &*

Karasawa 1953) and in the rat (*Imamura* 1959 a). This coincides with an extensive new formation and differentiation of the secondary nodules in lymph nodes and white splenic pulp When this differentiation of the secondary nodules has been completed, the number of mitochondria decreases

The mitochondrial contents of blood lymphocytes in rabbits with lymphatic hyperplasia produced by daily intravenous injections of ovalbumin have been measured by *Awaya & Tagawa* (1956), and by *Awaya* (1956) after only one injection The repeated daily injections resulted in a marked lymphocytosis and a significant rise in the average number of mitochondria in the blood lymphocytes with maximal changes 7 days after the initial injection The single injection of ovalbumin was followed by a transient increase of mitochondria, reaching a maximum after 3 days and then decreasing These investigations were repeated and entirely confirmed in the albino rat by *Imamura* (1959 c) He concluded that a hyperplasia of the lymphatic tissue, and especially of the secondary nodules stands in relation to a marked elevation in the average number of mitochondria in the blood lymphocytes This inference derived support from his investigations on albino rats exposed to x-rays (*Imamura* 1959 b) A single dose of 600 r sufficed to produce severe destruction of the lymphocytes and especially of the secondary nodules Immediately after the irradiation, a drop was noted in the blood lymphocyte count and in the average number of mitochondria in the lymphocytes In the course of regeneration of the lymphatic tissue, there was a striking transient elevation of this mitochondrial number which coincided with an extensive new formation of the secondary nodules during the period between the 14th and 22nd day after irradiation

Among the various hormones that influence lymphoid tissue and lymphocytes, cortisone is found to cause a fall in the mitochondrial contents of the blood lymphocytes (*Monden, Kanesada & Fukutani* 1956) This fall is very abrupt, a minimum value being attained 3 hours after the injection On the 5th day the mitochondrial contents have returned to normal in spite of the fact that the absolute number of lymphocytes in the blood has failed to regain its normal value

The purpose of the present investigation is to examine the influence of thyroxin on the blood lymphocytes with special reference to their mitochondrial contents, and to correlate these observations with earlier findings concerning the histologic picture of lymphatic hyperplasia induced by thyroxin treatment every third day for 20 days

MATERIAL AND METHODS

47 male and female guinea pigs weighing 300 400 g were employed 11 of these animals were used as untreated controls and the rest were divided into 6 groups with 6 animals in each group The animals in the 6 groups were treated with thyroxin

every third day from the onset of the experiment A dose of thyroxin of 5 micrograms per 100 g body weight was injected subcutaneously into the back of the animals in the same way as in an earlier work (*Fraström & Gyllensten* 1959).

The animals in the 6 groups were killed by a blow in the neck on the 1, 3, 6 9, 16 and 20 day after the first thyroxin injection, and the control animals at different times during the experimental period The cervical and extremety lymph nodes were dissected quantitatively and weighed by a technique described by *Glimstedt* in 1936 The thyroid, thymus, spleen and adrenals were also removed and weighed. The amount of white cells per mm³ of venous blood was registered in the usual manner in a Burker chamber The relative number of mononuclear and polynuclear cells was measured

Immediately after an animal had been killed, a small drop of blood from the right cardiac ventricle was stained supravitally with Janus green B and neutral red according to *Culling* in the Handbook of Histopathological Technique The slide was placed in 37° C for 10 minutes and then examined in a light microscope at 1000 × [illegible]

[illegible] und to be actively moving and their nuclei were transparent A count of 50 cells had to suffice in a few of the animals owing to necrobiotic changes in the cells

The lymph [illegible]

[illegible] number of mitochondria in the lymphocytes of each animal was calculated

It was as a rule easy to record the [illegible]
lympho [illegible]
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RESULTS

The two test groups containing removed lymph nodes disclosed, in all the thyroxin-treated animals, an increase in weight calculated in milligrams of lymph node mass per *100 g body* weight The relative weight of the thyroid decreased in the animals treated with thyroxin The weights of the *spleen*, *adrenals* and thymus varied insignificantly

TABLE 1

Differential Counts of Lymphocytes in the Blood of Thyroxin-Treated Guinea Pigs

Days after first thyroxin injection	Number of guinea pigs examined	Number of lymphocytes examined	Classification of lymphocytes according to their mitochondrial contents Lymphocytes in per cent						Average number of mitochondria $\bar{x} \pm e_{\bar{x}}$
			0-5	6-10	11 15	16-20	21-30	31-40	
Controls	11	1100	21 2	45 6	22 9	7.2	2 6	0 5	9 4 ± 0 5
Day + 1	5	450	16 9	46 4	27 3	7 6	1 8	0 0	9 7 ± 0 7
+ 3	6	600	13 0	34.2	34 8	12 8	5 0	0 2	11.3 ± 0.3
+ 6	6	600	6 8	22 8	27 7	22 0	14 0	6 7	15 4 ± 0 7
+ 9	6	550	8 5	22 2	33 8	21 1	11 1	3 3	14 2 ± 0 4
+ 16	6	533	11 6	37.3	30 6	12 2	6 2	2 1	12 0 ± 0 4
+ 20	6	550	13 1	41 5	27 4	11 1	5 1	1 8	11 3 ± 0 5

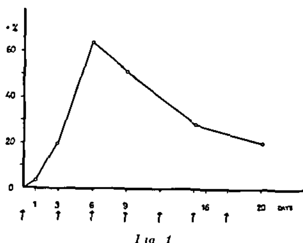


Fig 1
Increase in per cent in the average number of mitochondria per blood lymphocyte in guinea pigs after treatment with thyroxin (thyroxin injections every third day)

The mean values of the white blood cells varied but slightly above and below the corresponding mean value of the control animals The relative number of mono- and polynuclear cells in the blood was unchanged

The mitochondrial contents of the blood lymphocytes in the various groups of experimental animals were found to be increased when compared to those of the control animals (Table 1) The differences were analysed by the chi-square method

As soon as 1 day after the first thyroxin injection the blood lymphocytes showed a rise in the mitochondrial contents This increase was highly significant 3, 6, 9, 16 and 20 days from the start of the experimental period (p less than 0 001) When the mean values of the average number of mitochondria per lymphocyte in the different groups of animals were compared, a continuous rise was noted during the first half of the experimental period with a maximum on the 6th day when the average number of mitochondria per lymphocyte gained 64 per cent as compared to the control animals During the second *half* of the experimental period this average number diminished but remained throughout far above that of the control animals At the end of the experiment the average number of mitochondria exceeded that of the *control animals by 20 per cent (Fig 1) The reduction in the mito*chondrial contents of the blood lymphocytes from the 6th to the 20th day was markedly significant (p less than 0 001)

DISCUSSION

The influence of thyroxin on the lymph nodes thymus and spleen ascertained in the present investigation is consistent with earlier results arrived at by others (*Gyllensten* 1953) It has been convincingly established on a more comprehensive material than ours *that the* thyroxin-induced gain in weight of the lymph nodes is statistically

highly significant while the changes in the spleen and thymus are not significant The fact that thyroxin administration causes a reduction in thyroid weight has been demonstrated earlier and has been attributed to a compensatory decrease in the secretion of the thyreotropic hormone from the pituitary gland

The present investigation has proved that the greatly accentuated proliferation of lymph node tissue caused by the thyroxin is reflected in the blood by a striking increase in the contents of mitochondria in the lymphocytes This corresponds to findings in lymphatic hyperplasia induced by other means Thus, this increase of the mitochondria may probably be assumed to be an indication of intensified production of lymphocytes in the lymph nodes causing a larger number of young circulating blood lymphocytes The daily rise in the average number of mitochondria was, moreover, maximal from the 3rd to the 6th day after the start of the thyroxin treatment, and during the same three day-period the daily increase in the weight of the lymph node tissue was also maximal (*Ernstrom & Gyllensten* 1959)

When the growth promoting effect of the thyroxin treatment diminishes after about a fortnight, owing perhaps to compensatory mechanisms causing reduced production of endogenous thyroxin, there is a simultaneous decrease in the average number of mitochondria per lymphocyte

Further the incre[illegible]
may be due directly [illegible]
tissue and the lymp[illegible]
known to stimulate the oxygen consumption in most tissues, such an hypothesis is not improbable, seeing that this increase in oxygen consumption coincides with the development of the lymphatic hyperplasia *Gyllensten* (1953) treated young guinea pigs with thyroxin, using the same doses and the same intervals as in the present investigation He measured the oxygen consumption in groups of animals and found an increase of about 13 per cent during the period between the 4th and 10th day after the introduction of thyroxin treatment as compared to groups of control animals On the 14th day the oxygen consumption was little above that of the normal animals (*i e* 2 9 per cent) From this it may be inferred that the average number of mitochondria per blood lymphocyte reflects not only the growth processes in the lymph node tissue but also the oxygen consumption of the whole animal

It emerges from our observations that during thyroxin treatment there is an increased supply of lymphocytes with high mitochondrial contents to the blood, probably of young cells As there is no increase in the total number of blood lymphocytes, an intensified disappearance of lymphocytes from the blood may be conceived Whether these lymphocytes are used as stem cells in the bone marrow for increased production of red blood cells (see *Yoffey & Courtice* 1956, *Harris* 1960),

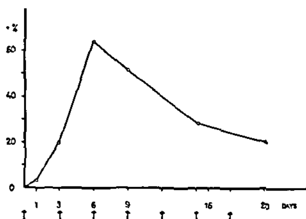


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When the growth promoting effect of the thyroxin treatment diminishes after about a fortnight owing perhaps to compensatory mechanisms causing reduced production of endogenous thyroxin there is a simultaneous decrease in the average number of mitochondria per lymphocyte

Further the increase in the mitochondria in the blood lymphocytes may be due directly to an added oxygen consumption in the lymphatic tissue and the lymphocytes delivered to the blood As the thyroxin is known to stimulate the oxygen consumption in most tissues such an hypothesis is not improbable seeing that this increase in oxygen consumption coincides with the development of the lymphatic hyperplasia *Gyllensten* (1953) treated young guinea pigs with thyroxin using the same doses and the same intervals as in the present investigation He measured the oxygen consumption in groups of animals and found an increase of about 13 per cent during the period between the 4th and 10th day after the introduction of thyroxin treatment as compared to groups of control animals On the 14th day the oxygen consumption was little above that of the normal animals (i e 2 9 per cent) From this it may be inferred that the average number of mitochondria per blood lymphocyte reflects not only the growth processes in the lymph node tissue but also the oxygen consumption of the whole animal

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or they are used generally as a source of nucleoproteins for the body tissues which have an increased metabolic rate induced by thyroxin, are both questions that remain to be elucidated, being intimately connected with the still unsolved problem of the significance of the lymphocyte

Though there is a considerable proliferation of pyroninophilic cells in the lymph node marrow during thyroxin treatment, it is most likely that most of the increase in the weight of the lymph nodes can be attributed to an absolute growth of the cortical layers and an increased production of lymphocytes

SUMMARY

The effect of thyroxin (5 micrograms per 100 g of body weight every 3rd day for 20 days) on blood lymphocytes in guinea pigs was examined by means of mitochondrial counts in lymphocytes stained supravitally with Janus green B and neutral red

The mitochondrial contents of the blood lymphocytes were significantly increased during the period from the 3rd to the 20th day after the first thyroxin injection A maximal increase of the average number of mitochondria per lymphocyte was obtained on the 6th day Then the mitochondrial contents again decreased, remaining above those of the control animals throughout the period of treatment

The correlation between the mitochondrial contents of the blood lymphocytes and the growth processes in the lymph node tissue and the oxygen consumption in the thyroxin-treated animals were discussed

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MALIGNANT LESIONS AFFECTING THE BILE DUCTS IN THE POST-MORTEM CHOLANGIOGRAM

By

KÅRI A. LARSEN

Received 21. vii. 60

Malignant diseases often involve the liver and its hilar region changing the course of the bile ducts both inside and outside the liver. Primary malignant lesions arising in the organs surrounding the bile ducts are not as frequent as metastases, but together they make up a fairly large group with difficult diagnostic problems. By means of new or improved roentgenological methods it is possible discover changes in the *clinical cholangiogram* fairly characteristic of *malignant lesions* in and near the bile ducts. The most important methods in this respect are the following: 1) Peroral cholangio- and cholecystography. 2) Intravenous cholangio- and cholecystography. 3) Cholangio- and cholecystography during operation (by direct injection). 4) Cholangio- and cholecysto-graphy through laparoscope (by direct injection) (Royer). The 3 first methods mentioned are daily used in most roentgen-departments, while the last is not widely employed.

The litterature on these methods is rich (*Rudström, Norman, Frommold, Evans et al.* and *Wise & O'Brien*) but most of the illustrations shows the difficulty of demonstrating details in the clinical cholangiogram. The best pictures gives the direct injection *during operation* or *through laparoscope*, and these methods may give the same quality as post-mortem cholangiograms.

The *post-mortem cholangiogram* can in many cases show a good picture of the extension of a malignant lesion in the liver and the hilar region, and make up the background for interpretation of the clinical cholangiogram.

We have analysed *4000 consecutive autopsies* performed in the Department of Pathology, Ullevål Hospital, Oslo during the period 1954–56. The autopsy material consists of about 40–50 per cent of all deaths in Oslo during the same period. This gives us a good idea of the incidence of the various lesions, *although an autopsy series always has* to be a selected material.

The autopsy material is also examined by post-mortem cholangiography in order to find pathologic changes, and these changes has

been compared with the gross pathology In order to get different cholangiographic pictures cases from lated periods are also used (Up to 1958)

The malignant lesions which are of great importance in producing changes in the form and course of the bile ducts are shown in Table 1

TABLE 1

Number among 4000 Autopsies—1954–56

Pr mary mal gnant t imours n th l	18 cases
	5
	79 —
	385 —
infiltrations)	116

The occurrence of the primary malignant tumours of the liver and the bile ducts is as expected Primary malignant tumours of the pancreas are comparatively frequent 1 50 This may be explained by the fact that we took a particular interest in these disease in the period mentioned However it may also be interpreted as a previous under estimate of the frequency of malignant pancreatic tumours The incidence of metastases to the liver is in accordance with that found in large materials of liver biopsies 1 10 (*Ward Ulevitch & Schiff*)

PRIMARY MALIGNANT LESIONS OF THE LIVER

In large autopsy materials from Europe and North America these tumours occur in a frequency of 2 3 per thousand In extensive series covering long periods of time there seems to be a slight increase in the incidence of carcinomas of the liver during the later decades both absolute and compared with other forms of cancer (*Kohn*) Kohn s material comprises 85 cases of primary cancer of the liver in Berlin in the period 1930 53 In the last part of the period there was a statistically valid increase in the frequency compared with the first part of it —The largest recently reported material of primary cancer of the liver is present by *Berman* from South Africa In a mon — ree

Edmondson & Steiner (1954) in the period 1918 53 found 100 primary carcinomas of the liver among 489000 autopsies performed at Los Angeles County Hospital There were [illegible] Of the last 7 [illegible] ological chang[illegible] 1) No dular [illegible] ducts (8 2) Massive 19 3) Diffuse tumours 3 Seven of the nodular tumours were near the hilus hepatic and their size was from 2 to 10 cm This classification may be useful when explaining the various changes in the cholangiogram

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ULLEVÅL SYKEHUS OSLO NORWAY*

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Received 21 vii 60

Malignant diseases often involve the liver and its hilar region, changing the course *of the bile ducts both inside and outside the liver*. Primary malignant lesions arising in the organs surrounding the bile ducts are not as frequent as metastases, but together they make up a fairly large group with difficult diagnostic problems. By means of new or improved roentgenological methods it is possible discover changes in the *clinical cholangiogram* fairly characteristic of malignant lesions in and near the bile ducts. The most important methods in this respect are the following: 1) Peroral cholangio- and cholecystography 2) Intravenous cholangio- and cholecystography 3) Cholangio- and cholecystography during operation (by direct injection) 4) Cholangio- and cholecysto graphy through laparoscope (by direct injection) (Royer). The 3 *first methods mentioned are daily used in most roentgen-departments* while the last is not widely employed.

The litterature on these methods is rich (*Rudström, Norman, Frommold, Evans et al* and *Wise & O Brien*) but most of the illustrations shows the difficulty of demonstrating details in the clinical cholangiogram. The best pictures gives the direct injection during operation or through laparoscope and these methods may give the same quality as post-mortem cholangiograms.

The *post-mortem cholangiogram* can in many cases show a good picture of the extension of a malignant lesion in the liver and the hilar region, and make up the background for interpretation of the clinical cholangiogram.

We have analysed *1000 consecutive autopsies* performed in the Department of Pathology Ullevål Hospital Oslo during the period 1954 56. The autopsy material consists of about 40 50 per cent of all deaths in Oslo during the same period. This gives us a good idea of the incidence of the various lesions although an autopsy series always has to be a selected material.

The autopsy material is also examined by post-mortem *cholangiographs* in order to find pathologic changes and these changes has





Fig 1

Upper Case 1 *Mal* ... located and curve ... some of the periph ... irregularly runn ... ca... s and curving Compression near the hilar region In the left lobe normal bile ducts

In nodular forms which are not too extensive, the dislocation of the intrahepatic bile ducts is caused by the individual nodule, and the changes are particularly pronounced in the peripheral ducts. In the massive forms both the peripheral and the central ducts show dislocation, and it is usually much more marked than in the nodular forms. The same is the case in the diffuse forms, here with even still more extensive changes.

In all forms the localization and the histological appearance are of primary importance, these factors being determinant of the affection of the bile ducts. Centrally located tumours, especially those near the hilus hepatis often produce compression of the large intrahepatic bile ducts with dilatation of the corresponding peripheral ducts. The varying richness of cells and stroma in the tumours certainly influences the development of the compression.

Among our 4000 autopsies we have observed in all *18 cases with primary malignant lesions in the liver*. The average age of these patients was 63 years. 11 of them were males, 7 females. The histological classification was as follows: Malignant hepatomas 12, Malignant cholangiomas 5, Sarcoma 1. In 10 of the patients the tumours had occurred in a cirrhotic liver. In the remaining 8 cases there was no definite cirrhosis. Two of the latter group were young patients, and two of them had extensive malignant lesions throughout the liver, the condition of the remaining liver tissue being eclipsed by the tumour masses. The four remaining cases of the non-cirrhotic group had either a central cholangioma (3) or a malignant hepatoma of the right lobe (1).

The *size of the greatest tumours* in the liver is shown in Table 2.

TABLE 2

The Size of Primary Tumours of the Liver (The Largest Single Tumour is Recorded)

< 5 cm	< 10 cm	> 10 cm
3 cases	3 cases	12 cases

It is apparent that most of the primary malignant tumours of the liver are fairly large when the subject comes to autopsy, and the lesions are prone to bring about marked changes in the cholangiogram.

The localization of the tumours in the liver is of primary importance. Among our 18 cases 5 had tumours near the hilar region with possibilities of *involvement* of the central bile ducts. In one of the cases the tumour was located distinctly in the periphery causing only small affection of the peripheral bile ducts. Among the 18 cases 12 had the tumour in the righ lobe and 1 in the left lobe. 5 cases had tumour masses in both lobes.

The change in the cholangiogram will best be elucidated by four cases.



Fig 9

Upper Case 3 Malignant hepatoma The central bile ducts in the hilar region are compressed with enormous dilatations in the peripheral ducts—Lower Case 4 Malignant cholangioma peripheral tumor Remarkably small changes in the cholangiogram only some irregularities in the course of the bile ducts upper left

Case 1 Male, 71 years old Malignant hepatoma Cirrhosis of the liver In the right lobe of the liver there were two tumours one the size of an orange and the other the size of a mandarin, as well as scattered metastases elsewhere in the parenchyma

The cholangiogram (Fig 1) revealed that some of the peripheral bile ducts in the right lobe were dislocated and bent forming large oval spaces between the ducts The more central ducts in this area were compressed showing curve shaped impressions in the outlines of the ducts In the right lobe there were some marked dilatations peripheral to the compressed ducts

Case 2 Female 20 years old Malignant hepatoma In the right lobe of the liver there was a lobulated round tumour with a diameter of about 20 cm

The cholangiogram (Fig 1) revealed irregularly running bile ducts in the right lobe normal conditions in the left lobe The right-sides bile ducts were dislocated and bent, particularly in the central areas of the lobe The ducts were partly wider than normal Close to the hilar region the ducts seemed to be somewhat compressed The whole picture did not give the impression of one single tumour but of several smaller ones

Case 3 Female, 86 years old Malignant hepatoma In the right lobe of the liver close to the midline there was a firm fibrous round tumour with a diameter of about 10 cm with scattered small metastasis in the surroundings The peripheral bile ducts were considerably dilated

The cholangiogram (Fig 2) revealed a central compression of the ducts 1 2 cm long The compression was pronounced In the left lobe the ducts were very dilated in some areas moderately widened in others In the right lobe there was moderate dilatation of most of the ducts The typical bending and dislocation of the ducts was not seen clearly although their course was irregular

Case 4 Female, 61 years old Malignant cholangioma In the right lobe of the liver there was a round tumour with a diameter of 6–8 cm located laterally and downwards as well as small scattered metastase in the surroundings

The cholangiogram (Fig 2) revealed remarkably small changes considering the size of the peripheral tumour Only the most peripheral bile ducts in the area involved showed some irregularitiets in their course with an unusually wide space between the surface of the liver and the peripheral bile ducts

In cases 1 and 3 the diagnosis of expanding process should not be difficult Differential diagnosis from a cyst is called for in case 1, but the form of the dislocation and the compression of the central bile ducts should point to a tumorous infiltration If it were a liver cyst the bile ducts around the expanding process would probably be much wider

In case 2 the process seems to involve the larger part of the right lobe and the dislocation of the bile ducts is in favour of tumours of middle size It is difficult to differentiate this picture from that of metastases The only feature that might help is that there are no changes in the cholangiogram in the left lobe

Case 4 demonstrates that a fairly large tumour does not necessarily cause considerable changes in the cholangiogram when it is located peripherally Fortunately this is comparatively rare of our 18 cases only one had this location

The *changes in the cholangiogram* which seem to be most important in cases of primary malignant tumours of the liver are the following

(1) Fairly large irregular dislocations and bending of the bile ducts particularly in the right lobe



Fig 2

Upper Case 3 *Malignant hepatoma* The central bile ducts in the hilar region are compressed with enormous dilatations in the peripheral ducts *Lower* Case 4 *Malignant cholangioma* peripheral tumor Remarkably small changes in the cholangiogram only some irregularities in the course of the bile ducts upper left

(2) Compression of the bile ducts, particularly in the central area near the hilus. Secondary dilatations of the more peripheral ducts.

PRIMARY MALIGNANT LESIONS IN THE EXTRAHEPATIC BILE DUCTS

(Including Carcinoma of the Papilla of Vater)

These tumours are comparatively rare. In great autopsy series we can expect about 1:4000 primary carcinomas in the bile ducts outside the liver, when the lower end of the choledochus is not included (*Stewart, Lieber & Morgan*). *Puestow, Wurtz & Olander* found 11 cases of carcinoma of the papilla of Vater among 5765 patients with malignant tumours. Among our 4000 autopsies there were *5 cases of primary carcinoma of the extrahepatic bile ducts* (the gall bladder not included). One of these cases originated from the papilla of Vater. The other four cases had carcinoma of the common bile duct (2 patients), of the main hepatic duct (1 patient) and of the cystic duct near the common bile duct (1 patient).—The average age of the patients was 71 years. Four of them were females, one of them male.

The characteristic feature of these carcinomas is the comparatively rapid development of occlusion of the duct affected. Generally the bile ducts peripheral to the occlusion are dilated due to stasis of the bile. Infection is a frequent complication. The occlusion of the bile ducts is often extensive, and injection of contrast substance from the gall bladder or ductus choledochus will probably not give good visualization of the peripheral bile ducts, the contrast flowing into the duodenum instead of passing the stricture.

In two of the five cases we have been able to make cholangiograms.

Case 5. Female 78 years old. Carcinoma of the hepatic duct. At the site of the junction in the hilus of the liver there was a hard nodular tumour about the size of a hazel nut, narrowing the ducts to an extreme degree.

The cholangiogram (Fig. 3) revealed free passage of the contrast medium into the common bile duct, which was of normal width. In the hepatic duct there was only little contrast, and there were irregular, ragged outlines at the site of the partition and 2–3 cm further down. The contrast did not pass the stricture.

Case 6. Male 57 years old. Carcinoma of papilla of Vater. At the papilla there was a tumour about the size of a hazel nut with ulceration of the duodenal mucosa. The tumour caused a stenosis of the lower part of the ductus choledochus.

The cholangiogram (Fig. 3) revealed a narrowing of the lower part of the common bile duct only sufficient for the passage of a knitting needle. The duct widened upwards like a funnel and the upper part of the common bile duct and the hepatic duct were considerably dilated. The outlines of the stenosed portion were somewhat irregular. The crooked outlines on both sides showed the growth of the tumour above the lower part of the common bile duct.

The *characteristics of the cholangiogram* in cases of tumours originating in the extrahepatic bile ducts and of the ampulla of Vater are the following:



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lower part f the common bile duct —*Lower left* Case 7 *Care nom of the hea l the pancreas* Mo lerate c mj ression of the common b le duct and the major pa cr at c duct w th d latations *per j herical to the sten s s*—*Lower r ght* Case *(re n ma f the hea l of the j ancreas* Marked irregular str cture of the comm b le duct cl e t the papilla f Vater Tumor impression n the duodenal l p

1) Stricture of the bile duct 2) Irregular outlines of the stricture, to a certain extent distinguishing the stricture from compression from the outside 3) Dilatation of the bile ducts above the stricture 4) In tumour of papilla of Vater the funnel-shaped outlines of the stricture and dilatation are quite characteristic and different from the stricture formed by benign and malignant tumours or fibrosis of the head of the pancreas

PRIMARY MALIGNANT LESIONS IN THE PANCREAS

Carcinoma is the predominating disease of the pancreas, and is also a fairly frequent lesion in comparision with carcinoma in other organs Usually carcinoma of the pancreas is estimated to account for 2–4 per cent of all deaths of cancer In the Cancer Registry of Norway the total number of new cases diagnosed in the two years 1953 54 as *malignant disease of the pancreas was 385 in a population of 3, 4 mill* In an autopsy material the incidence of carcinoma of the pancreas will be larger because there are fewer possibilities of diagnosing this disease outside hospitals

Among our 4000 autopsy cases there were *79 patients with carcinoma of the pancreas* The *average age was 67 years* There were 46 males and 33 females

In regard to the cholangiogram the most important carcinomas are those located in the head, and partly those in the body of the pancreas The close relation between the head of the pancreas and the lower part of the common bile duct explains the ease with which tumours in these regions cause compression Tumours of the body of the pancreas may also affect the common bile duct, the middle part of it passing close to the medial part of the body Involvement of the duct in these cases will not take place before the tumour extends beyond the organ Tumours of the tail of the pancreas rarely affect the bile ducts

The localization of primary malignant tumours within the pancreas is shown in Table 3

TABLE 3

Localization of Carcinoma of the Pancreas

Localization	Number of cases	Number of cases with compression of the common bile duct
Caput pancreatis	33	30
Corpus pancreatis	29	15
Cauda pancreatis	10	0
Local not recorded	7	3
	79	48

As expected the affection of the common bile duct is found most frequently with primary carcinoma of the head of the pancreas As these tumours grow there is hardly any case without more or less compression of the duct Often a direct infiltration of the wall of the duct is observed Even the mucosa may be involved, although that is comparatively rare

In cases with *carcinoma of the body compression of the common* duct was recorded in half of the cases As experience shows, the middle part of the common bile duct is particularly involved in these cases The compression appears only after the tumour has infiltrated the surroundings

In cases with carcinoma of the tail we have not seen any definite affection of the common bile duct by direct extension of the tumour

In *the cholangiogram* there will be two types of compression, according to the localization of the carcinoma

1) Compression of the lower part of the bile duct in its course through the head of the pancreas 2) Compression of the middle part of the common bile duct in its course near the body of the pancreas — The degree of compression may of course vary In the late stage seen at autopsy the compression is often marked To a certain degree the compression is in proportion to the size of the tumour, but in the head this is not necessarily the case A table show roughly the recorded size of the primary tumour at the autopsy

TABLE 4

The Size of the Primary Pancreatic Tumour at Autopsy
(in 32 Cases)

	< 5 cm	6–10 cm	> 10 cm
Number of cases	10	15	7

As shown more than ⅔ of the tumours are more than 6 cm in diameter In the head this often results in marked compression and in the body in extensive infiltration of the surrounding tissue possibly causing stricture of the middle part of the common bile duct

The findings in the cholangiogram in these cases will be demonstrated by some cases

Case 7 Male 82 years old Carcinoma of the head of the pancreas The tumour in th[illegible] [illegible] compressed the lower part of the [illegible] with dilatation of the duct systems [illegible]

[illegible] cholangiogram was hardly characteristic but it demonstrated a moderate stricturing process in the head of the pancreas

Case 8 Female 65 years old Carcinoma of the head of the pancreas The tumour measured about 5 cm in diameter and compressed the common bile duct causing a

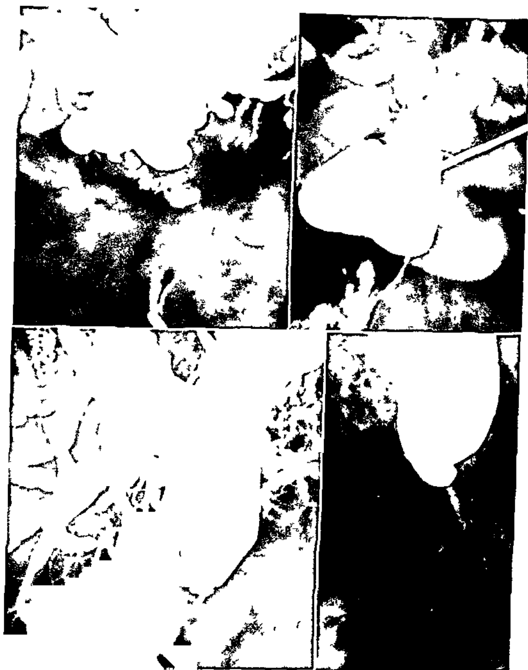


Fig 5

Upper left Case 9 *Carcinoma of the head of the pancreas* Irregular stricture of the common bile duct with moderate dilatation above The major pancreatic duct is strictured with peripheral dilatation Both strictures have irregular ragged outlines indicating malignant infiltration —*Upper right* Case 10 *Carcinoma of the head of the pancreas* In the lower part of the common bile duct there is a stricture with enormous dilatation above —*Lower left* Case 11 *Carcinoma of the head and body of the pancreas* Pronounced and irregular stricture of the lower part of the common bile duct Irregular outlines Enormous dilatation above the stricture *Lower right* Case 12 *Carcinoma of the head and body of the pancreas* In the lower part of the common bile duct there is a long irregular narrow stricture with enormous dilatation above

stricture 1.5 cm long close to the papilla Vateri. Anastomosis between the common bile duct further up and the duodenum had been done. The common bile duct and the cystic duct were very dilated.

The cholangiogram (Fig. 3) revealed a marked, irregular stricture of the common bile duct close to the papilla of Vater. The duct was moderately dilated above the stricture, and due to the anastomosis the duodenum was filled with contrast. In the duodenal loop there was a large impression caused by the tumour.

Case 9. Male, 58 years old. Carcinoma of the head of the pancreas. The tumour measured about 5–6 cm i diameter. It was hard and nodular, and infiltrated the lower part of the common bile duct save of the very last cm of the common duct. Both the common and cystic bile ducts were markedly dilated.

The cholangiogram (Fig. 4) revealed an irregular stricture of the common bile of about 3–4 cm in length. Above the stricture the duct was moderately dilated. Also the major pancreatic duct was filled with contrast and was stenosed for about 4 cm with dilatation peripheral to this part. Both strictures were irregular and ragged indicating malignant infiltration.

Case 10. Male, 73 years old. Carcinoma of the head of the pancreas. The tumour measured about 5 cm in diameter. It compressed the lower part of the common bile duct for about 2 cm of its length. The lumen was only wide enough to admit a knitting needle, and the common bile duct and the cystic duct were considerably

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Case 12. Male, 69 years old. Carcinoma of the head and body of the pancreas. ...
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Case 13. Female, 91 years old. Carcinoma of the head and body of the pancreas. The tumour measured about 4 cm in diameter. ...
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Case 14. Female, 71 years old. Carcinoma of the body of the pancreas. The tumour measured about 8 cm in diameter. The hard tumorous infiltration had extended to the surroundings. The common bile duct was ...

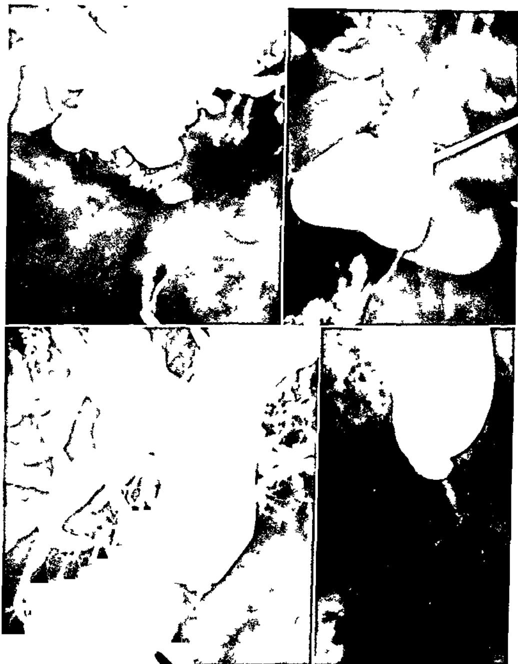


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Case 10 Male 73 years old Carcin ma of the head of the pancreas The tumour measured al out 5 cm in d ameter It compressed the lower part of the common l ile duct f r ab ut 2 cm of ts length The lumen was nly w de enough to adm t a kn tting needle an l the c mmon l le duct and the cystic duct were consideral ly d lated al ove

The cholang ogram (F g 4) revealed a stricture of the lowest part of the common b le duct about 2 cm in lenght Mark l d latation of the duct al ove the str ct re

Case 11 Female 80 years 11 Carc noma f the head and l ody of the pancreas

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Case 12 Male 69 years ol l Carcinoma l the hea l an l body of the pancreas In the hea l and bod of the pancreas there was a large tum ur measuring ab ut 7×10 cm It cau e l an irregular long str cture of the common l ile duct The tumour infiltrate l the luct wall alth ugh the mucosa was annar ntl

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Case 13 Female 91 years old Carc noma of the head and body f the pancreas The tum r mea ure l al out 4 cm n diameter The hard t m ro s nf ation s c mpre s n of ater The c mm n and cystic

The ch lang ogram
al e the papilla f Va 3 cm
t e duct ab ve the les on the cystic b le du t an l the gall l la l l r were consi lerably d lated

Case 14 Female 71 years ld Carc n ma of the l ody of the pancreas The tumour mea ured al ut 8 cm in d ameter The hard tum rous infiltration had extended to the urr und ng The common l le duct was inf ltrated and strictured in the middle part w th pr nounced d latation al ve

The ch langi gram (F g 5) reveale l a short irregular stricture of the middle part

The most important *route of the tumour spread* is via *the portal vein, and in particular tumorous emboli from the gastrointestinal tract* spread into the adjoining organs in this way. Tumorous emboli from other organs may spread via the *hepatic* artery, especially from the *breast, lungs and kidneys.* More rarely the lymphatics serve as the route of tumour spread. In theses cases the spread is retrograde from the liver hilus. The portal vein distribution favours the transportation of carcinomatous metastases from the stomach, pancreas or ascending *colon into the right lobe* and from the sigmoid or rectum into the *left lobe,* according to the streamlines of flow in the portal vein system (*Popper & Schaffner*).

In our material of 4000 autopsies *metastases of the liver were found in 385 patients.* 204 were males, 181 females.*The average age was 65 years.*

The primary seat of the carcinoma, giving rise to liver metastases, is of great interest. The various origin of the malignant lesion is shown in Table 5 Some special types of tumours are grouped separately.

TABLE 5

The Primary Seat and Incidence of Tumours Giving Rise to Liver Metastases (385 cases)

(Primary Tumours of the Liver and Bile Duct System not Included)

Carcinomas (according to primary seat)		*Special type of malignant tumours* (according to type)		*Carcinomas and undifferentiated malignant tumours with unknown primary seat*	
Stomach .	75	Malignant melanoma	6	Total	15
Colon rectum	50	Reticulosarcoma	6		
Breasts	48	Malignant neurinoma	4		
Pancreas	38	Hodgkins disease	4		
Bronchial tree, lungs	31	Myeloid leukemia	3		
Uterus (Corpus & cervix)	15	Carcinoid tumours in stomach-intestine	3		
Prostate	14	Lymphatic leukemia	2		
Ovaries	*12*	*Lymphosarcoma*	2		
Oesophagus	11				
Kidneys	11				
Gall bladder	10				
Urinary bladder	7				
Testes	5				
Skin lips	3				
Small intestine	3				
Rare primary seats	7				

In Table 5 it is shown that the metastases from carcinomas originating in the stomach, intestines and pancreas formed the most important group Together they constitute close on half of all metastases of the liver The next most important group is formed by carcinomas from the breasts and the genitals. A fairly large group is composed of metastases from carcinomas in the bronchi and lungs The other types of tumours are either rare or seldom give metastases to the liver The metastases

from tumours originating in mesenchymal tissue make up considerable number when added together although they are not nearly as frequent as the metastases from carcinomas

The size and amount of the metastases vary considerably From scattered microscopic tumours there may be every transition to nearly complete tumorous infiltration throughout the whole liver The weight of the liver with metastases may be far more than double the normal *Sherlock* refers to a patient with metastases to the liver which was said to weight 21 5 kg

Based on the statements in the records the weight of the liver and some photographs we have tried to estimate the amount and size of the liver metastases at the time of death The result is shown in Table 6 and 7

TABLE 6

The Amount of Metastases at Autopsy (385 Patients)

Solitary	Scattered	Numerous	Innumerable nodule or thoroughly infiltrated liver
43	118	74	150

As shown in Table 6 the two last groups are the largest It is surprising that in 150 patients there were enormous masses of metastasis and in 74 patients there were numerous metastases in the liver It is evident that extensive changes must profoundly affect the *cholangiogram* particularly in the peripheral intrahepatic bile ducts

TABLE 7

The Diameter of the Metastases in the Liver at Autopsy (385 Patients)

< 3 cm	3-6 cm	7 12 cm	> 12 cm (and confluent tumorous masses)
140	145	61	39

In Table 7 it is shown that the metastases are mainly less than 6 cm in diameter 285 cases having metastases of that size The large tumours were found in the remaining 100 cases the huge masses in 39 of them

In the cholangiogram the greatest changes will be noted in cases with the large tumours Tumours measuring less than 6 cm in diameter will probably also cause dislocation of the peripheral bile ducts in a great many cases but here the changes will be more difficult to interpret if they are not very extensive

By *post mortem* injections of contrast medium we have been able to demonstrate slight changes *in the cholangiogram* even in metastases measuring about 3 cm in diameter In cases with tumours measuring 3 6 cm in diameter apparent changes were often found but probably not so marked that it would always be of diagnostic aid in the cholangiogram *in vivo*

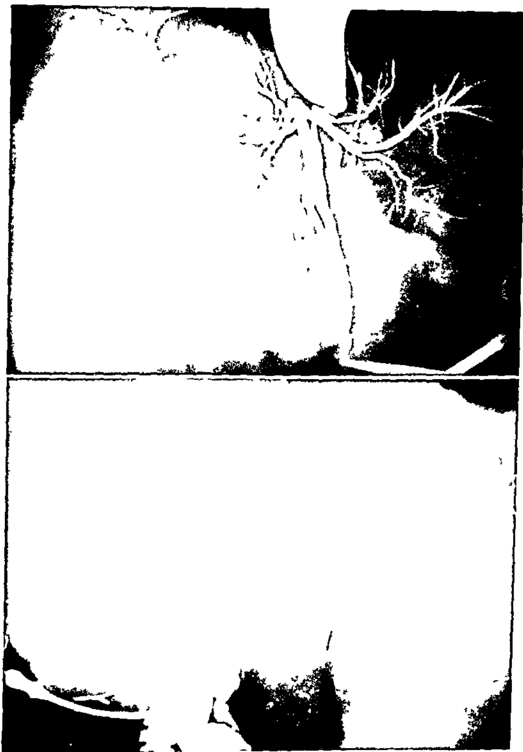


Fig 6

Upper Case 16 *Carcinoma of the rectum with metastases in the liver especially in the right lobe* —The bile ducts in the right lobe are irregularly distributed They are elongated and considerably curveshaped with impressions and flattenings In the left lobe the ducts appear normal apart from a slight wavy form in one of them
Lower Case 17 *Carcinoma of the stomach with metastases to the whole liver* The bile ducts are elongated and there are marked curveshaped impressions and flattenings They are partly spread apart partly closely packed Some of the peripheral ducts are slightly dilated

The metastatic tumours measuring about 6 cm or more in diameter cause marked changes particularly in the peripheral bile ducts At autopsy we observed most of the tumours situated between the larger bile ducts In cases with large tumours the bile ducts pierce right through some of the tumorous masses In general we may expect that some of the metastases will dislocate and kink the larger bile ducts We would also expect compression of the bile ducts with flattening of the lumina Peripheral dilatation of the bile ducts in these cases is seldom seen at autopsy

Among the post mortem cholangiograms which we have performed we have selected some of those with particularly marked changes, caused by large and extensively spreading metastases

Case 16 Males 62 years old Carcinoma of the rectum with liver metastases In the right lobe of the liver the metastatic tumours measured up to 8–10 cm in diameter while in the left lobe they were few and small

The cholangiogram (Fig 6) [illegible]
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and flattenings The tributarie [illegible]
hepatic ducts there was a curv [illegible]
tributaries seemed to be a little dilated In the *left* lobe the ducts appeared normal apart from a slightly wavy form in one of them

Case 17 Female 83 years old Carcinoma of the stomach with metastases to the liver The metastatic tumours in the liver were enormous and only little normal liver tissue was left

The cholangiogram (Fig 6) revealed that some of the intrahepatic bile ducts were elongated and had marked curve shaped impressions The tributaries were irregularly distributed partly closely packed partly spread apart In some of the peripheral ducts there were some slightly dilated sections

When the changes are as obvious as they were in these two cases, they will probably also be easily observed *in vivo* The severely impaired liver function in such cases will probably make it difficult to produce a cholangiogram by intravenous contrast injection However, in some cases with large metastases the liver function may be fairly good making it possible to demonstrate the tumours even by intra venous cholangiography By direct injection the changes should be readily visible

The most important *changes in cases with liver metastases* are the following 1) Fairly extensive curve shaped dislocations of the intra hepatic bile ducts 2) The changes are spread over large areas 3) Irregular distribution of the peripheral bile ducts 4) Only slight dilatations of the peripheral bile ducts

TUMOUROUS INFILTRATION AND METASTASES IN THE LIVER HILUS

At autopsy of cases with malignant tumours we often [illegible]
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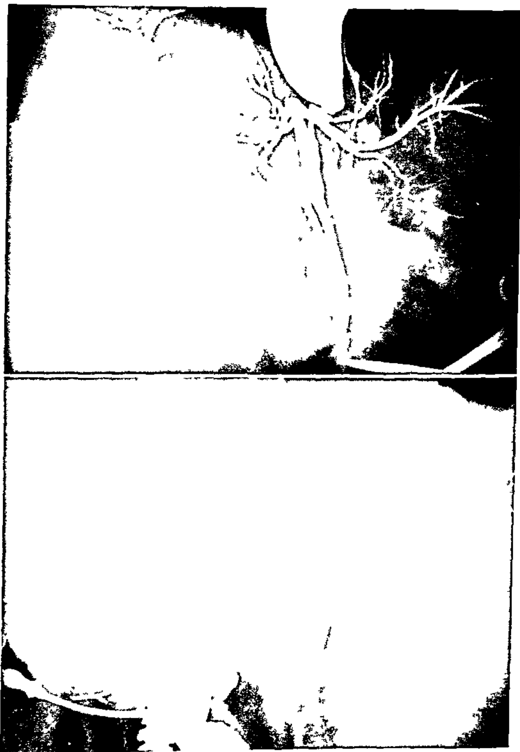


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The cholangiogram (Fig 6) revealed that some of the intrahepatic bile ducts in the *right* lobe were considerably elongated and showed curve shaped impressions and flattenings The tributaries were irregularly distributed In one of the central hepatic ducts there was a curve shaped impression and flattening and one of the tributaries seemed to be a little dilated In the *left* lobe the ducts appeared normal apart from a slightly wavy form in one of them

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The most important *changes in cases with liver metastases* are the following 1) Fairly extensive curve shaped dislocations of the intrahepatic bile ducts 2) The changes are spread over large areas 3) Irregular distribution of the peripheral bile ducts 4) Only slight dilatations of the peripheral bile ducts

TUMOUROUS INFILTRATION AND METASTASES IN THE LIVER HILUS

At autopsy of cases with malignant tumours we often find metastatic infiltration in the lymph nodes of the liver hilus and in the surrounding tissue The malignant infiltration reaches the hilar region by

several routes: 1) Direct extension from tumours in the neighbouring organs. 2) Lymphatic spread. 3) Intraperitoneal or hematogenous spread.

Direct extension from tumours in neighbouring organs, such as the stomach, pancreas and gall bladder, is probably the most important. Such tumorous infiltration may be very massive, surrounding the extrahepatic bile ducts and cause compression of varying degree. *The lymphatic spread* may originate from several organs and tumour types: a) From tumours in the liver. They are comparatively rare, but in large percentage they metastasize to the regional lymph nodes. b) From secondary tumours (metastases) in the liver. This is a large group, and lymphatic spread from these metastases is probably very frequent. c) From primary tumours in the stomach. These are important in view of the frequency of these tumours. d) From secondary tumours in the retroperitoneal lymphatic system, especially in the upper abdomen. Carcinomas of the pancreas, intestines and other abdominal organs often spread to the upper retroperitoneal lymph nodes, whence retrograde spread to the liver hilus may occur. *The intraperitoneal or blood stream* spread is probably rare.

Metastatic tumour infiltration in the hilar lymph nodes usually gives rise to enlargement of the nodes, but this enlargement rarely cause any marked compression of the extrahepatic bile ducts. We have in some cases observed such compression in our autopsy material, and in these cases the lymph nodes were very large. The greatest compression are caused by tumorous infiltration outside the lymph nodes. The bile duct was in these cases fixed by infiltration in the wall, or they were surrounded by tumorous tissue. Compression without fixation of the duct is seldom pronounced. The jaundice in these cases may be produced by compression of the extrahepatic ducts, but in most cases it is probably due to the liver metastases causing destruction and degeneration of the liver tissue itself (*Popper & Schaffner, Sherlock*).

In our autopsy material *116 cases out of 1000 had tumorous infiltration in the hilar tissue or metastases with enlargement of the lymph nodes in this region*. Among these 116 cases more or less compression of the extrahepatic bile ducts was recorded in 65 cases. The *average age of the 116 cases was 65 years*. 66 of them were males, 50 females. As we see the average age was the same as in the group with liver metastases.

The origin of the tumours was mostly in the neighbouring organs. This may indicate direct tumorous infiltration or massive propagation along the lymphatics. The various origins of the tumours are shown in Table 8.

TABLE 8

Origin of Metastases Liver Hilus Tumours

Carcinomas (according to primary seat)		Spec. types of mal. tumours (according to type)		Carcinomas and undiff. mal. tumours with unknown primary seat	
Stomach	45	Malignant neurinoma	3	Total	5
Pancreas	18	Reticul. sarcoma	2		
Intestines	12	Hodgkin's disease	2		
Genitals (both sexes)	9	Malignant melanoma	1		
Breasts	5				
Gall bladder	4				
Bronchi lungs	4				
Primary carcinomas of the liver	2				
Oesophagus	2				
Rare prim. seat	2				

In Table 8 is shown that the principal origins of the malignant infiltration in the liver hilus are the stomach intestines and pancreas. Primary tumours in the liver and gall bladder give rise to some metastatic lesions in this region. Considering the incidence of the myeloid and lymphoid tumour forms they constitute a fairly large number.

The *secondary liver tumours* (385) among our 4000 autopsies are not recorded in Table 8. However as many as 80 cases among the 116 patients had liver metastases. We suppose that many of the cases in Table 8 first got their liver metastases and the infiltration in the liver hilus was secondary to them.

In the cholangiogram mainly the extensive tumour infiltration may be of importance. The infiltrations which directly extend into the hilar region from a neighbouring organ we have already demonstrated in the chapter on carcinomas of the pancreas. In several cases of metastases to the hilar region we have been able to inject contrast substance and in many of them we have found compressions of the extrahepatic ducts of greater or lesser degree. Three illustrative cases will be reported.

Case 18. Male 61 years old. Carcinoma of the small intestine. The lymph nodes in the retroperitoneal space and the liver hilus were greatly enlarged due to tumour infiltration. Enlarged nodes in the hilar region compressed the common bile ducts moderately.

The cholangiogram (Fig. 7) revealed apparent impressions in the common b[illegible]

Case 19. Males 60 [illegible]
there [illegible]
ing [illegible]
The [illegible]
and [illegible]
compr[illegible] [illegible]patic duct were not clearly shown but they did not seem to be dilated.

Case 20. Female 66 years old. Carcinoma of the colon. In the hilus of the liver there was tumorous infiltration both in the lymph nodes and in the hilar tissue

several routes 1) Direct extension from tumours in the neighbouring organs 2) Lymphatic spread 3) Intra-peritoneal or hematogenous spread

Direct extension from tumours in neighbouring organs, such as the stomach, pancreas and gall-bladder, is probably the most important Such tumorous infiltration may be very massive, surrounding the extra-hepatic bile ducts and cause compression of varying degree *The lymphatic spread* may originate from several organs and tumour types a) From tumours in the liver They are comparatively rare, but in large percentage they metastasize to the regional lymph nodes b) From secondary tumours (metastases) in the liver This is a large group, and lymphatic spread from these metastases is probably very frequent c) From primary tumours in the stomach These are important in view of the frequency of these tumours d) From secondary tumours in the retroperitoneal lymphatic system, especially in the upper abdomen Carcinomas of the pancreas intestines and other abdominal organs often spread to the upper retroperitoneal lymph nodes whence retrograde spread to the liver hilus may occur The *intraperitoneal or blood stream* spread is probably rare

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The origin of the tumours was mostly in the neighbouring organs This may indicate direct tumorous infiltration or massive propagation along the lymphatics The various origins of the tumours are shown in Table 8

partly forming a hard fixed infiltration The common bile duct was compressed and infiltrated and the duct above was not dilated

The cholangiogram (Fig 7) revealed compression of the common bile duct in the upper part The compressed portion measured 1½ cm in length and the outlines were irregular in the narrowed lumen The major pancreatic duct was filled and showed a similar narrowing of the lumen at the same distance from the papilla of Vater

It is surprising that the dilatation of the ducts peripheral to the compressions in this case was not more marked than that shown However the compressions of the ducts were clearly seen

It is very important to have in mind all the tumour forms dealt with here when interpreting cholangiograms The secondary tumours adjacent to the bile ducts may not have the same therapeutic interest as the primary ones but the incidence of the secondary tumours is so great that we have to take them into consideration each time malignant infiltration is assumed

SUMMARY

Malignant diseases in the liver the hilar region of the liver the pancreas and the papilla of Vater often affect the bile ducts with compressions displacements strictures and dilatations These lesions of the bile ducts have been studied in the *post mortem cholangiogram* and correlated with the autopsy findings both in the specimens in which the cholangiograms were performed and in an autopsy series of 4000 consecutive autopsies

Among the 4000 autopsies we found 1) Primary malignant tumours *in the liver* 18 cases 2) Primary malignant tumours in the extra hepatic *bile ducts* 5 *cases* 3) Primary malignant tumours in the pancreas 79 cases 4) Metastases to the liver 385 cases 5) Metastases to the hilar *region of the liver* (in the lymph nodes or diffuse infiltrations) 116 cases

Post mortem cholangiograms of *illustrative cases of these lesions* are demonstrated and the different lesions of the intra and extra hepatic bile ducts are pointed out Special emphasis is laid on the secondary malignant tumours *(metastases) in the liver* and the hilar region of the liver because of their great number and the changes wrought in the cholangiogram which are often marked

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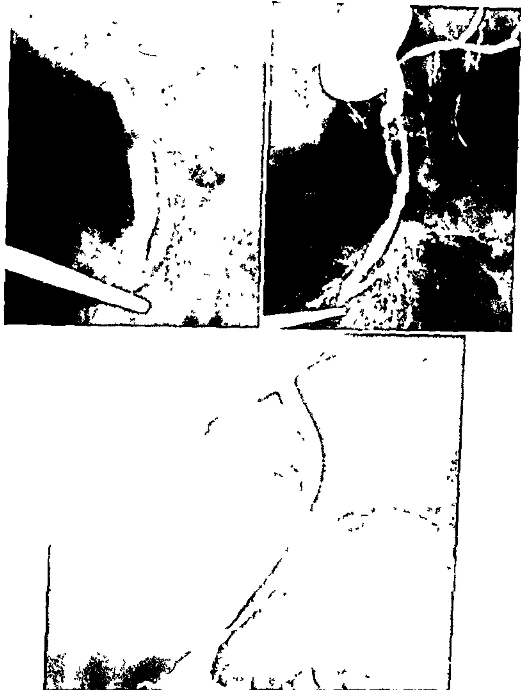


Fig. 7

Upper left. Case 18. *Carcinoma of the small intestine.* In the common hepatic duct and ductus choledochus there are small impressions which are somewhat irregular. The lymph nodes in the liver hilus were severe enlarged due to tumor infiltration. *Upper right.* Case 19. *Carcinoma of the stomach with tumor infiltration in the liver hilus.* The cystic duct and the common hepatic duct are moderately compressed. The cystic duct above the compression are dilated. The hepatic ducts above the compression shows little dilatation. *Lower.* Case 20. *Carcinoma of the colon with tumor infiltration in the liver hilus and retroperitoneal in the pancreatic region.* Ductus choledochus is compressed in its upper part with irregular outlines in the narrowed lumen. The major pancreatic duct is also filled and shows a similar narrowing of the lumen in the same distance from the papilla of Vater. The dilatations above the tumor strictures are surprisingly moderate.

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BENIGN LESIONS AFFECTING THE BILE DUCTS IN THE POST MORTEM CHOLANGIOGRAM

By

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In a previous paper the author has dealt with *malignant* lesions affecting the bile ducts in the post mortem cholangiogram and showed the different changes in these lesions (Larsen). The *benign lesions* affecting the bile ducts are not so often described apart from the gall stone disease (*Rudström, Bassler & Peter, Norman*).

With the more extensive use of different cholangiographic examinations the varying benign diseases affecting the bile ducts will be encountered more frequently. Consequently it may be of interest to be familiar with the changes in the post mortem cholangiogram in these cases, even if this not always can be identical with the changes in the clinical cholangiogram.

At *Ullevål Hospital, Department of Pathology* we have a fairly large autopsy material comprising between 40 and 50 per cent of all deaths in Oslo. This should give a good picture of the incidence of the different benign diseases. We have not dealt with gall stone disease as most of the questions relating to this malady have been thoroughly canvassed.

Apart from gall stone disease the most important benign lesions which may affect the bile ducts are the following: (1) Hepatic cysts (2) Haemangiomas (3) Cirrhosis of the liver (4) Pancreatitis and fibrosis of the pancreas.

During a period in 1954 to 1956 we have examined in all 4000 consecutive autopsy records with special regard to the diseases mentioned. In several cases we have had the opportunity to make *post mortem cholangiography*, most of them with *barium sulphate* as contrast medium. In this way we were able to demonstrate various pathological changes in the cholangiogram due to benign lesions.

The incidence of the different diseases in the 4000 consecutive autopsy cases is shown in Table 1.

As appears from this table hepatic cysts are comparatively frequent. This is probably due to the high average age in the autopsy material (67 years). The number of haemangiomas seems to be somewhat low judged by the impression we get in our daily work in the autopsy room.







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Probably we have to allow for several small haemangiomas not recorded or overlooked

TABLE 1

	Number among 4000 consecutive autopsy cases (1954–56)
Hepatic cysts	36 patients
Hepatic haemangiomas	16 –
Hepatic cirrhosis	126 –
Pancreatitis and fibrosis of the pancreas	26 –

The number of cirrhosis is what we should expect The cases with pancreatitis and fibrosis of the pancreas are very difficult to judge Some of the reported cases of pancreatitis have only necrosis and bleeding and we know that this may occur, at least to a moderate degree during the dying period or they may be post-mortem changes We have included all cases in which the diagnosis could be made with certainty

HEPATIC CYSTS

The hepatic cysts are amply treated in the literature on pathology, above all by *Moschowitz* who gathered 85 cases from the literature and and added 6 in 1906 Later on they are thoroughly dealt with by *Meyenburg* in 1918 He described also groups or clusters of small bile ducts in the liver lobules separated from the portal areas These have since been known as Meyenburg's complexes He interpreted these as persisting intrahepatic bile ducts that fail to involute, and cysts result from their gradual cystic dilatation This concept of the origin of polycystic liver has been supported by many authors *Melnick* found in the autopsy records of the Los Angeles County General Hospital 70 cases of polycystic liver recorded over a 36-year period —an incidence of 1 per 687 autopsies The condition was increasingly frequent with advancing age There was a high incidence of association with bilateral congenital polycystic kidneys and occasionally with cysts of the pancreas, lungs and other organs

Cysts of other types such as parasitic, hamartomatous, teratoid and other cysts are of less importance They are not found in the present autopsy material

Fig 1

Upper *Case 1* Large hepatic cyst in the left lobe —Distended hepatic ducts around the cyst not dilated

Middle *Case 2* Large hepatic cyst in the left lobe —The distended hepatic ducts are dilated

Lower *Case 3* Hepatic cyst in the left lobe The hepatic ducts were not distended There was a marked dilatation of the ducts in the left lobe and it could give impression of a central stenosis







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The total number of *patients with hepatic cysts* among our 4000 autopsies amounted to 36. They were solitary in 17 patients, multiple in 19. The size of the cysts is indicated in Table 2.

TABLE 2

The Size of Hepatic Cysts (The Largest one Is Recorded)

< 3 cm diameter	18 patients
3– 6 – –	13 –
7–12 – –	3 –
> 12 –	2 –

As shown, most of the cysts are of moderate size. The majority of them would probably not cause any marked changes in the bile ducts, while the larger ones do cause alterations *as demonstrated in Fig. 1*. Several of the cysts 3–6 cm in diameter may be shown if they are favourably located. All cysts more than 6 cm in diameter are likely to appear in the cholangiogram if not situated very unfavourably in the liver.

The greater frequency of hepatic cysts in the *present material* (1 : 111) in comparison with that of *Melnick* (1 : 687), is probably due to the advanced *average age of all our cases at autopsy (67 years)*. The average age in the patients with *hepatic cysts was 75 years*. 15 males and 21 females make up the material.

Six of the hepatic cysts were examined microscopically. The wall of the cysts was composed of a comparatively thin fibrous membrane covered by flat epithelium, which in places seemed to form several layers. In large cysts the epithelium was particularly flattened. Branches *of the hepatic ducts lying close to the cysts were often somewhat dilated*. In one of the cases the cysts were only slightly *distended and the* cholangiogram revealed wide bile ducts which could falsely indicate a more central stenosis.

The changes in the cholangiogram are made clear by three selected autopsy cases.

Case 1. Female 86 years old. Hepatic cyst. Fig. 1.

In the left lobe of the liver a distended serous cyst the size of a large orange was found.

The cholangiogram of the liver reveals bile ducts bending around the large cyst in the left lobe. The ducts are disproportionately long in comparison with those of the right lobe. A few of the ducts in the left lobe are wider than normal.

Case 2. Male 61 years old. Hepatic cyst. Fig. 1.

In the left lobe of the liver a distended serous cyst the size of a large orange was found.

The cholangiogram shows a *medium sized cyst in the left lobe. The bile ducts* bend around the cyst. The ducts are remarkably wide in comparison with those of the right lobe, although no stenosis in the main trunks was noted.

Case 3. Female 77 years old. Hepatic cyst. Fig. 1.

In the left lobe of the liver several serous cysts were found. The largest cyst was the size of a fist. The cysts were not distended and were rather limp.

The cholangiogram displays wide ducts with an unusual course. They are dislocated with wide interspaces particularly near the hilus. They show partly marked dilatation and one may get the impression of stenosis in the main trunks. The comparatively slight distension of the serous cysts probably causes the unusual picture.

The most important roentgenological features in these cases are: 1) Smooth dislocated intrahepatic bile ducts with cystic interspaces. 2) Somewhat wide and elongated intrahepatic bile ducts. 3) No signs of stenosis in the larger trunks, the hepatic or the common bile duct.

HAEMANGIOMAS

Most haemangiomas are small and of little importance. To a larger degree they are casual findings at autopsy, and they rarely give rise to any symptoms. However, they may cause severe haemorrhage, necessitating surgical intervention.

Most haemangiomas show cavernous arrangement of thinwalled endothelial lined spaces filled with blood. The classifications of these tumours may be difficult, and some authors do not consider them as tumours (*Willis*). The differentiations from venectasia is sometimes difficult.

We have recorded in all *16 patients* with haemangiomas of the liver among our 4000 autopsy cases. In 4 cases there were multiple haemangiomas, in 12 cases they were solitary.

The size is given in Table 3.

TABLE 3

The Size of the Haemangiomas in 16 Patients

(the Largest Haemangiomas Is Recorded if Multiple)

< 2 cm in diameter	10 patients
2-4 – –	5 –
5-8	1 –

The *average age was 65 years*. The material consisted of 13 males and 3 females.

We had the opportunity to inject contrast and to study the postmortem cholangiogram in the patient with the largest haemangiom.

Case 4. Female 69 years old. Haemangioma of the liver. Fig. 2.
In the left lobe of the liver a bluish gray tumour measuring about 4×4×6 cm was found.
The cholangiogram revealed elongated bile ducts bending around an oval area with out contrast filled ducts. The ducts were not particularly dilated at least not in proportion to the length. The ducts followed the contour of the haemangioma and there was no considerable dislocation of the ducts with wide interspaces as seen in the cystic livers.

The *X ray picture in cases of haemangiomas* does not show any conclusive feature. The only thing that should be mentioned is that bile ducts bend around the haemangioma and not pierce through it, and that the ducts follow the contour of the tumour.



Fig. 2

CIRRHOSIS OF THE LIVER

The most important forms of cirrhosis are recorded fairly frequently in our autopsy material. The usual type *portal cirrhosis* is ordinarily due to virus hepatitis, alcoholism or other intoxications, but the terminal picture may be similar or even indistinguishable whatever the aetiology is. The next important type is *cardiac cirrhosis*, which has another pathogenesis, but also this type may be similar to the portal cirrhosis late in the disease.

The unusual forms, such as *Bilharzia cirrhosis*, *haemochromatosis and biliary cirrhosis*, are of little importance when considering the bile ducts changes in the cholangiogram. Of course biliary cirrhosis is of consequence but this diffuse fibrosis is eclipsed by the primary changes in the larger bile ducts. We will only deal with cirrhosis of the *toxic infectious group* and the *cardiac group*.

We had in all *126 patients* with cirrhosis of the two groups among our 4000 autopsies, 62 were females, 64 males. The *average age was 68 years*. The distribution of the patients in the two groups is shown in Table 4.

TABLE 4

Cirrhosis of the liver

Toxic infectious group (94)			*Cardiac* (32)		
Slight changes	Moderate changes	Pronounced changes	Slight changes	Moderate changes	Pronounced changes
13	14	67	21	10	1

The *toxic infectious group* is the largest one and cases with pronounced changes predominate. The pathogenesis in these cases is primarily a massive damage of liver cells causing more or less necrosis among them. The reticulin framework collapses with approximation of portal and central zones. The liver cells are replaced by irregular bands of fibrous tissue and surviving cells regenerate to form nodules of various sizes. Fibrous septa develop in the portal zones and in the lobular parenchyma. The distribution of the fibrous septa may vary with the causative lesion. In the cases with well marked changes the fibrosis is extensive and there is a shrinkage of the whole organ. One of the most striking features histologically is the proliferation of the small bile ducts, a trait that is the object of much discussion. Both

---

Fig. 2

Upper: *Case 4*. Hepatic haemangioma in the left lobe.
Middle: *Case 4*. Cholangiogram of the same hepatic haemangioma with moderate filling of the hepatic ducts. It shows the bile ducts around the haemangioma on one side.
Lower: *Case 4*. The same haemangioma with further injection of contrast dye. It shows deposits of contrast dye outside the liver. The bile ducts can be seen surrounding the haemangioma on both sides.



Fig 5

The cholangiogram shows a small liver with nodular contours. The bile ducts run close together and their course is irregular and finely wavy. The larger trunks are somewhat dilated.

In conclusion we may state that the *post mortem cholangiographic picture of cirrhosis of the liver* may be fairly characteristic, particularly when the changes are advanced with marked shrinkage. In our autopsy material the advanced forms are very frequent in the toxic infectious group but not in the cardiac group. The advanced forms of liver cirrhosis of the toxic infectious group are total more than half of all the recorded liver cirrhosis in our autopsy material and we may assume that these forms are often met with also in clinical materials.

The most important changes are: (1) Finely wavy course of the bile ducts. (2) Close irregularly running bile ducts in a small liver. (3) Dilatation of the larger intrahepatic ducts in the most advanced cases. (4) With water soluble contrast filling of the small peripheral bile ducts may succeed and they show some dilatation. Possibly also the proliferating small bile ducts may be observed.

Pancreatitis and Fibrosis of the Pancreas

There are several aetiological and pathogenetic problems concerning pancreatitis. It is difficult to find a reasonable classification on macropathological and histological grounds. The post mortem changes in autopsy materials complicate the matter. For practical purposes in connection with the influence on the bile ducts we have used the following classification: (1) Acute and subacute interstitial pancreatitis. (2) Acute and subacute haemorrhagic and necrotic pancreatitis. (3) Fibrosis of the pancreas.

The *acute and subacute interstitial pancreatitis* is characterized pathologically by interstitial oedema, cellular infiltration consisting of granulocytes and lymphocytes, as well as areas of fat necrosis. If the process is extended and involves the caput, compression of the common bile duct may be the result.

Acute and subacute haemorrhagic and necrotic pancreatitis is charac

Fig. 8.

Upper: Case 7. — the b[illegible]e ducts w[illegible]

Lower: Case 8. [illegible] al [illegible] [illegible] el w th [illegible] bile ducts are dilated. [illegible] parts of the bile ducts are not filled. The central



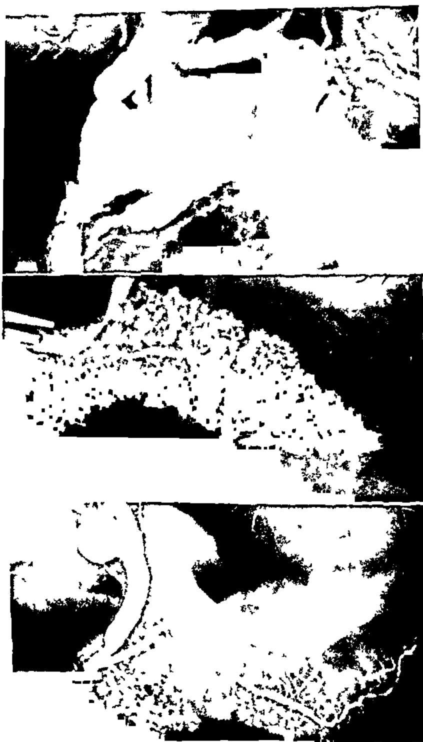


Fig 5

dilated above the stenosis. The compression occurring in cases of fibrosis of the pancreas is often of quite another character than the compression in cases with tumours. In fibrosis the walls of the common bile duct are pressed together although the passage is comparatively sufficient, at least as long as there is no active inflammation of the duct. The deformation of the common bile duct may be demonstrated in the cholangiogram, especially when it is well marked.

Compression of the common bile duct was found in 3 of the 9 cases with acute and subacute interstitial pancreatitis. Also in these cases we have to consider a certain failure in the observation of the compression Probably most of the cases with affection of the caput have a certain degree of compression

In cases with acute and subacute haemorrhagic and necrotic pancreatitis the pathological condition in the pancreas predominates to such an extent that a possible *compression of the bile duct will not be* noticed. We have recorded compression in 2 of the 5 cases, but we have no contrast injections in these cases. Included in these form of pancreatitis we have to expect *a few cases of infarction due to arterial embolia.*

In cases of pancreatitis and fibrosis of the pancreas we have succeeded in producing useful cholangiograms·

Case 9 Male, 70 years old Subacute interstitial pancreatitis Fig 5
Pancreas particularly the caput, was enlarged There were areas of fat necrosis The lower part of the common bile duct was narrowed Histologically there was a subacute, interstitial inflammation The cholangiogram shows a moderate narrowing of the lower 2–3 cm of the choledochus

The stenosis is fairly even and circular Contrast substance in duodenum reveals an irregular impression by the caput into the duodenal lopp There is retrograde filling of the major pancreatic duct, which does not seem to be particularly dilated

Case 10 Male, 46 years old Chronic, recurring pancreatitis with fibrosis Fig 5
Pancreas of normal size, firm, very hard in places When out it appeared indurated The common bile duct was moderately narrowed with a slight dilatation above the pancreas The cholangiogram and pancreatogram reveal that the choledochus is *compressed in the lower part to an extent of 3–4 cm assuming the form of the sheath* of a sword The outlines of the narrowed duct are even and regular The common bile duct above the deformed part is not considerably dilated The major pancreatic duct is somewhat wider than normal The small, peripheral pancreatic ducts are well *filled and they are ectatic* all over the pancreas

Case 11 Female, 55 years old Chronic pancreatitis with fibrosis Fig 5
The pancreas was nodular and hard especially in parts of the caput When cut it appeared to be indurated No stenosis of the choledochus was observed

The cholangiogram and the pancreaticogram showed slight stenosis of the common bile duct in the lower 1 1½ cm *The stenosis* was even The major pancreatic duct was of normal calibre In the lower portion of the caput there were several ectatic peripheral small ducts

The changes in the cholangiogram in the pancreatic diseases mentioned are not at all specific The only sign which may point to a marked fibrosis of the head of the pancreas is *the compression of the choledochus* which takes the form of the sheath of a sword, as in case 10

This sign is so characteristic that it together with the clinical findings allows the diagnosis of pancreatic fibrosis to be made

The compression in case 9 with subacute interstitial pancreatitis is far more difficult to differentiate from other pathological processes However the even compression with the regular outlines suggest a benign stenosis

The changes in the pancreatograms are not expected to appear without direct injection into the major pancreatic duct However this procedure may be very opportune during operation of cases where the differential diagnosis between a malignant tumour and fibrosis of the pancreas cannot be settled In such cases the only thing to be felt can be the firm partly hard pancreas and even a biopsy may be confusing An injection of contrast into the pancreatic duct may give a characteristic picture

In *conclusion* the benign stenosis due to the pancreatic conditions mentioned is of a different character from that of any malignant stenosis but there will always be cases with stenosis in an early stage in which it is impossible to decide the nature of the process

The *supporting features in favour of a benign lesion* are as usual
e narrowing of the lumen
part into the duct imme
duct above the stenosis
(5) In some cases characteristic contour of the stenosed part

SUMMARY

Benign diseases in the liver and the pancreas often affect the bile ducts with strictures dilatations and irregular course of the bile ducts These lesions of the bile ducts have been studied in the post mortem cholangiogram and correlated with the autopsy findings both in specimens in which the cholangiograms were performed and in an autopsy series of 4000 consecutive autopsies

Among the 4000 autopsies we found (1) Hepatic cysts in 36 cases (2) Hepatic haemangiomas 16 cases (3) Hepatic cirrhosis 126 cases (4) Pancreatitis and fibrosis of the pancreas 26 cases

Post mortem cholangiograms of illustrative cases of these lesions are demonstrated and different lesions of the intra and extra hepatic bile ducts are pointed out

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dilated above the stenosis The compression occurring in cases of fibrosis of the pancreas is often of quite another character than the compression in cases with tumours In fibrosis the walls of the common bile duct are pressed together although the passage is comparatively sufficient at least as long as there is no active inflammation of the duct The deformation of the common bile duct may be demonstrated in the cholangiogram, especially when it is well marked

Compression of the common bile duct was found in 3 of the 9 cases with acute and subacute interstitial pancreatitis Also in these cases we have to consider a certain failure in the observation of the compression Probably most of the cases with affection of the caput have a certain degree of compression

In cases with acute and subacute haemorrhagic and necrotic pancreatitis the pathological condition in the pancreas predominates to such an extent that a possible compression of the bile duct will not be noticed We have recorded compression in 2 of the 5 cases but we have no contrast injections in these cases Included in these form of pancreatitis we have to expect *a few cases of infarction due to arterial embolia*

In cases of pancreatitis and fibrosis of the pancreas we have succeeded in producing useful cholangiograms

Case 9 Male 70 years old Subacute interstitial pancreatitis Fig 5
Pancreas particularly the caput was enlarged There were areas of fat necrosis The lower part of the common bile duct was narrowed Histologically there was a subacute, interstitial inflammation The cholangiogram shows a moderate narrowing of the lower 2 3 cm of the choledochus
The stenosis is fairly even and circular Contrast substance in duodenum reveals an irregular impression by the caput into the duodenal loop There is retrograde *filling of the major pancreatic duct which does not seem to be particularly dilated*

Case 10 Male 46 years old Chronic recurring pancreatitis with fibrosis Fig 5
Pancreas of normal size firm very hard in places When cut it appeared indurated The common bile duct was moderately narrowed with a slight dilatation above the pancreas The cholangiogram and pancreatogram reveal that the choledochus is compressed in the lower part to an extent of 3 4 cm assuming the form of the sheath of a sword The outlines of the narrowed duct are even and regular The common
not considerably dilated The major pancreatic
The small peripheral pancreatic ducts are well
he pancreas

Case 11 Female 55 years old Chronic pancreatitis with fibrosis Fig 5
The pancreas was nodular and hard especially in parts of the caput When cut it appeared to be indurated No stenosis of the choledochus was observed
The cholangiogram and the pancreaticogram *showed slight stenosis of the common* bile duct in the lower 1 1½ cm The stenosis was even The major pancreatic duct was of normal calibre In the lower portion of the caput there were several ectatic peripheral small ducts

The changes in the cholangiogram in the pancreatic diseases mentioned are not at all specific The only sign which may point to a marked fibrosis of the head of the pancreas is the compression of the choledochus which takes the form of the sheath of a sword as in case 10

(FROM THE UNIVERSITY DEPARTMENT OF PATHOLOGY
ALLMÄNNA SJUKHUSET MALMÖ SWEDEN)

VASCULAR CHANGES IN CEREBRAL METASTASES

By

INGA HAGERSTRAND

Received 26 v 60

It has long been known that primary cerebral tumours occasionally show a curious vascular pattern. Even *Virchow* spoke of teleangiectatic gliomas. Attention has been given in particular to the endothelial proliferations. In tumours of the astrocytoma group the proliferation of the vessel walls has proved to vary with the grade of malignancy of the tumour (5 2). Vascular changes resembling those seen in malignant gliomas have also been observed in metastatic growths and in inflammatory processes of the brain (2 3) but have only received scanty space in the literature and the frequency and intensity of such vascular changes have not been studied in detail.

This paper is concerned with the occurence of vascular changes particularly endothelial hyperplasia in metastatic growths in the brain.

MATERIAL

The material was collected from cases autopsied between Jan 1 1957 and March 31 1960 at the University Department of Pathology Malmö Allmänna Sjukhus Malmö. During this period 3 3[illegible]8 autopsies had been performed and malignant tumours had been found in 139. In 54 of these cases the tumour had metastazed to the brain. Nine of them were not accepted for the present investigation because representative tissue specimens were no longer available. Of the remaining 45 cases 1 7 sections of cerebral metastases from each were studied. The sections [illegible]
in Table 1 [illegible]
addition 5 ca[illegible]
of my[illegible]
1 case of canc[illegible]
into the brai[illegible] [illegible] cancer of the skin of the face and 1 adenoid cystic cancer of the left maxillary sinus both with direct growth into the brain were examined. In none of these cases were any vascular changes observed.

FINDINGS

[illegible] o groups one (I) with
[illegible] id 7 They include what
[illegible] (3) These formations spread within the actual tumour tissue but as a rule not in necrotic regions. Changes showing signs of budding (Figs 4 and 5) were as

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1

Age in years	Number of metastases in: Cerebrum	Cerebellum	Endothelial proliferations: Perivascular intravascular formations	Budding formations	Large lacunar vessels	Hyaline thrombi
74	2	2	+		+	+
77	2	2		+		
55	3		+		+	
47	2		+	+		+
60	2					
61	1		+			
73	1		+	+	+	
57	1			"		
83	several		+	+	+	
63	2		+		+	+
84		1				
67	2			+		+
80	1		+			
80	several	1		+		+
63	1		+	+	+	+
57	2		+	+		+
64	several	1	+	+	+	+
34	"	several	+	+	+	
40	"	"				
57	1				+	
49	1	1			+	
52				+		+
66			+	+	+	+
60	2		+	+		
5	several		+	+	+	+
69			+	+	+	
53	"		+	+	+	+
67	"				+	
6	"	several	+		+	+
2	"			+	+	+
71	1		+		+	+
76	2				+	+
64	3		+			
65	3					+
81		1				
39	1	1			+	
77		3	+			
0	1		+		+	+
64	1					
23	2	several			+	+
62	1	1	+	+	+	
83	2					
84		1				+
47	several	1		+		
46	"	1	+		+	+

TABL

Site of primary tumour	Histological diagnosis	Sex
Lung	Adenocarcinoma	♂
,,	,,	♂
,,	,,	♀
,,	,,	♂
,,	,,	♂
,,	,,	♂
,,	,,	♀
,,	,,	♂
,,	Squamous carcinoma	♀
,,	,,	♂
,,	,,	♀
,,	,,	♂
,,	,,	♂
,,	,,	♂
,,	Oatcell carcinoma	♂
,,	,,	♂
,,	,,	♂
,,	,,	♂
Mammary gland	Carcinoma	♀
,, ,,	,,	♂
,, ,,	,,	♀
,, ,,	,,	♀
,, ,,	,,	♀
,, ,,		♀
Skin	Malignant melanoma	♀
,,	,,	♂
,,		♀
,,	,,	♀
,,	,,	♂
Kidney	Carcinoma	♂
,,	,,	♂
,,		♀
,,		♀
,,		♂
Stomach	Adenocarcinoma	♂
Large intestine		♂
,, ,,		♂
Rectum		♂
Liver	Hepatocellular cancer	♀
Testis	Choriocepithelioma	♂
Adrenal	Neuroblastoma	♂
Thyroid gland	Carcinoma sarcoma	♀
Pleura	Sarcoma	♀
Pleura	Mesothelioma	♂
Malignant tumour of unknown type and origin		♂



Fig 7
Endothelial proliferations within a tumour mass giving an angioma like picture
Htx eos × 100

signed to the second group (II) They correspond to what *Gough* calls glomerular formations (3) They occur in the periphery of the metastases but particularly in the contiguous brain tissue Transitional forms between the different types of changes are often seen Most of the tumours are very vascular Only in 2 cases was the blood supply scanty But some areas of endothelial hyperplasia were nevertheless demonstrable It is clear from the table that endothelial proliferations were observed in 30 out of 45 cases They included 5 cases with only vascular changes of the type seen in group II

No difference was found between the vascular changes in the mid brain and the cerebellum The endothelial proliferations did not appear to vary with the size of the metastases The most advanced and most extensive endothelial hyperplasia was seen in metastases from the lung cancers which represented two fifths of the material Also most of the cases of malignant melanomas showed proliferations of the vessel walls though they were not so marked as in cases of metastases from bronchial cancers No relation could be demonstrated with certainty between the endothelial changes and the site or histological type of the primary tumour It is also clear from the table that large lacunar vessels (Fig 6) were noted in 21 cases and hyaline thrombi (Figs 3 and 6) in 21 cases The latter vascular changes and endothelial proliferations in the cerebral metastases were as a rule by no means so marked

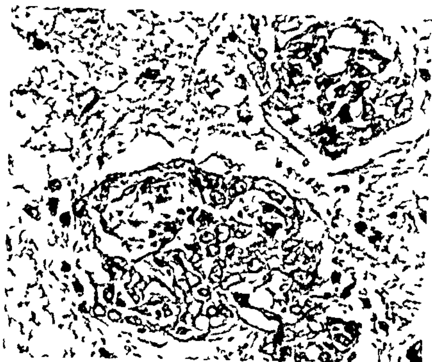


Fig 5
Budding formations Htx eos × 300



Fig 6
Large lacunar vessels and hyaline thrombus with scanty endothelial growth
Htx e s × 380



Fig 7
Endothelial proliferations within a tumour mass giving an angioma like picture Htx-eos × 100

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or extensive as in the cases of glioblastoma multiforme Of the 20 cases of glioblastoma examined, 16 showed abundant and 2 scanty endothelial proliferations In 17 cases there were large numerous lacunar vessels and in 18 hyaline thrombi sometimes associated with fibrinoid necrosis of the vessel walls

DISCUSSION

Neither the origin nor the cause of vascular hyperplasia is properly understood Some authors believe the condition to arise in the vascular endothelium, others in the pia-glial membrane of the vessels Most descriptions of malignant gliomas include both endothelial and adventitial proliferations (1) These changes have been ascribed, among other things, to healed hyaline thrombi (6) Though it is not possible to reject such an assumption, it is difficult to believe this to be the only cause of such hyperplasia Especially the budding phenomena appears to be due rather to an increased vessel new formation than to thrombosis of the vessels In the present material changes similar to those described by *Duguid & Storring* (6) were occasionally seen in the present cases but there was considerable disproportion between the incidence of vascular hyperplasia and hyaline thrombi This type of vascular proliferation, however, seems to be specific for cerebral tissue (2, 3) In the 45 cases described metastases outside the central nervous system were examined for proliferation of the vascular walls but in no instance were any such changes observed In regard to some of the cellular proliferations in vessels in glioblastoma it has hitherto been debatable whether they belong to the tumour tissue or not (4) The fact that similar proliferations often occur in metastatic epithelial tumours in which the tumour parenchyma can be readily distinguished from vascular proliferations clearly show that they are not neoplastic, but a specific reaction of the cerebral vessels to the tumour The fact that the vascular proliferations were *more numerous and more marked in the most* malignant gliomas may possibly be ascribed to an increase in the nutritional requirements of the rapidly growing tumour As to the metastatic tumours, no direct correlation could be demonstrated between the vascular proliferations and the grade of malignancy which was hardly to be expected as all metastatic growth must be regarded to show a high degree of malignancy

SUMMARY

Of 45 cases of metastatic cerebral tumours vascular proliferations of essentially the same type as those seen in more malignant types of gliomas were demonstrated in 30 No correlation was found between the site size and histological type of the tumours Vascular proliferations are manifestations of a *specific reaction of the cerebral vessels* since no such manifestations could be demonstrated in the primary

umours or in other organs The nature and causes of the vascular roliferations are discussed and it is suggested that they vary in extent with the rate of growth (nutritional requirements) of the tumour

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BACTERIUM ANITRATUM (ACINETOBACTER ANITRATUM) ISOLATED FROM A CASE WITH CEREBRAL ABSCESS AND PURULENT MENINGITIS

By

ROALD OPSAHL jr.

Received 28.viii.60

CASE REPORT

In the middle of March 1960 a 58 year old man was admitted to a neurosurgical ward with the diagnosis of tumor cerebri. At operation on March 24th a meningeoma was found in the temporal area. The tumor could not be completely removed and some neoplastic tissue along the ala ossis sphenoidalis had to be electrocoagulated. The first three days after operation he had complete rightsided hemiplagia and expressive aphasia, but he retained consciousness. The following days he improved slowly. April 4th his condition grew worse with rising temperature and in spite of treatment with Penicillin and Streptomycin, later Matromycin (Triacetyloleando mycin), he died April 18th. The post mortem examination showed a defect in the left temporal lobe where the tumor had been excised. This was connected with an abscess containing thin greyish green pus. The meninges at the base of the brain were covered with a yellow green pus layer.

Bacteriological Examination of the Pus

A gramstained smear showed an exudate rich in pus cells, among them gram negative organisms, mostly diplococci which were generally seen outside the cells. They appeared to be encapsulated.

Growth on blood agar: Greyish-white "fat", smooth colonies, round with a diameter of about one mm; they appeared to have a mucoid consistency when touched with the platinum loop. There was no change of the blood around the colonies. At first only these colonies were seen but after two days a few hemolytic colonies together with some swarming Proteus-colonies were observed on the original plate (see later). Growth in 1 per cent glucose broth: Abundant with a uniform turbidity. The organisms were non-motile.

Gram-stained smears from the culutures showed many coccoid diplobacilli, but with varying morphology from cocci to more clear-cut rods.

Acid was produced from glucose (no gas), arabinose and xylose but not from lactose, mannitol, maltose, saccharose and rhamnose within 5 days in fluid medium. Acid was produced on 10 per cent but not on 1 per cent lactose agar plates. These reactions were negative

Nitrate reduction, oxidase, urease (Christensen's medium), indol, Voges-Proskauer (Barrit's modification), methyl red H_2S and gelatin liquefaction The strain was non pathogenic to mice

After these tests one could establish the diagnosis of Bacterium anitratum Sensitivity tests showed that it was highly resistant to sulphonamides, penicillin, streptomycin, chloramphenicol, tetracyclin, oleandomycin nitrofurantoin bacitracin and növobiocin, relatively resistant to oxitetracyclin, chlortetracyclin, erythromycin and canamycin moderately sensitive to neomycin, and sensitive to polymyxin

In order to isolate the above mentioned hemolytic colonies, which were assumed to be grampositive, the growth from the primary plate was treated with ethyl ether for 30 seconds and subcultured on a new plate in order to get rid of the gramnegative organisms Unexpectedly, the gramnegative B anitratum grew abundantly after the ether treatment whereas the swarming and hemolytic colonies were killed The hemolytic colonies were then instead isolated from the original plate by spreading on a Sabouraud agar (to inhibit the swarming of the Proteus) Biochemical reactions showed that they were a hemolytic strain of Proteus The swarming colonies proved to be Proteus mirabilis

DISCUSSION

In this case it seems obvious that B anitratum was the cause of the infection of the brain and meninges The patient had probably been infected during the operation for a meningeal tumor The Proteus organisms were assumed to be postmortal contaminants since they were not seen by direct microscopy of the pus and since they grew very sparsely in the original culture

It seems that B anitratum is a microbe with a slight pathogenicity and which requires a generally or locally lowered resistance to be the cause of an infection *Waage* reported a case in 1952 where B anitratum was isolated from cerebral abscesses (1) It was then found in predominating numbers together with yellow hemolytic staphylococci and the role of B *anitratum* as a cause of the infection was uncertain Among Brooke's 86 strains of B anitratum (1951) (2) none was isolated from spinal fluid or the central nervous system *Piechaud & Second* in 1951 (3) reported 26 cases in which bacteria classified as Moraxella lwoffi had been isolated 10 of these appeared to be identical with B *anitratum* 5 of these strains had been isolated from spinal fluid 3 from fatal cases of postoperative meningitis, but it is not clear whether any of these 5 strains were B anitratum

The classification of this organism is a yet uncertain The fact that our strain was ether resistant contrary to the usual behaviour of the Enterobacteriaceae may be another piece of evidence that it should not be placed in this family (as was proposed by *Stuart et al* 1949) (4)

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Another strain of B. anitratum which was also ether resistant has since been isolated in this laboratory from the pus of a leg ulcer. *Brisou* (5) suggested that the organism should be referred to the genus Acinetobacter of Achromobacteriaceae, under the name of Acinetobacter anitratum. The microscopic picture may show a considerable resemblance to Neisseria organisms, as was remarked in this case. Because of this morphological similarity *De Bord* (6) considered the bacteria to belong to his tribe Mimeae of Bacteriaceae. It has not yet been classified in *Bergeys* manual (7. edition). Until an agreement is reached concerning the international nomenclature it seems convenient to use the name Bacterium anitratum, as was proposed by *Schaub & Hauber* (7) in their original description of this bacterium in 1948.

SUMMARY

A fatal case of postoperative cerebral abscess and purulent basal meningitis caused by B. anitratum is reported.

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STUDIES ON VARIANTS OF A BOVINE STRAIN OF PARAINFLUENZA 3 VIRUS

2 *Haemagglutinating Activity*

By

S HERMODSSON, Z DINTER and K BAKOS

Received 15 ix 60

The isolation of different variants from a bovine strain of parainfluenza 3 virus has been described in the first report of this series (5). The variants differed mainly in their cytopathic effect on calf kidney cultures and one of the variants seemed to produce less haemagglutinin In order to characterize these viruses more closely the haemagglutinating activity has been studied in some detail

MATERIALS AND METHODS

Tissue cultures of calf kidney cells were prepared as described in a preceding paper (5)

[illegible] produced a cytopathic [illegible] with active virus The [illegible] —20° C. [illegible] a 0 5 per cent suspension o[illegible] virus in 0 5 ml physiologic[illegible] formed by the red blood ce[illegible] for 2 hours The HA titre [illegible]

RESULTS

Haemagglutination Spectrum

The ability of the virus 23 and the variants 33 and 196 to agglutinate red blood cells from different species was found to be the same. They agglutinated human group O, bovine and guinea pig erythrocytes but as a rule not chicken, rabbit, sheep, goat or horse red cells. The HA titres were usually somewhat higher with guinea pig erythrocytes than with human or bovine erythrocytes.

Dependence upon Electrolytes and Influence of pH

Like other myxoviruses (4, 8, 11) parainfluenza 3 virus requires electrolytes for haemagglutination. This was demonstrated in an experiment in which red cells suspended in 0.15 M sucrose solution were added to dilution series of virus in 0.15 M sucrose or in 0.15 M sodium chloride solution.

In order to determine the influence of pH on the HA titer, packed red cells were suspended in appropriate buffers and added to dilution series of virus. Variation of pH in the range pH 5.5 to 9.0 had no significant influence on the HA titer. Similar results have been obtained with influenza virus (9).

TABLE 1

The Influence of Temperature on Haemagglutinating Activity

Virus	4° C	22° C	37° C a)				
	120 b)	120	5	15	30	60	120
23 stock A	16	64	32	64	64	128	64
23 stock B	16	256	128	256	256	256	256
33	32	512	256	256	512	512	512
196	16	128	32	128	128	256	256

a) After incubation at 37° C the titration series were placed at 4° C and read 2 hours after addition of erythrocytes.
b) Time in minutes.

Influence of Temperature

In order to investigate the influence of temperature on the HA titre, parallel titrations of the viruses 23, 33 and 196 were incubated with red cells at 4° C and 22° C for 2 hours and at 37° C for various periods. The results recorded in Table 1 show that the HA titre was 4 to 16 times higher after incubation at 22° C and 37° C than after incubation at 4° C. Furthermore the results in Table 1 show that the main effect had been produced within 5 to 15 minutes at 37° C. The increase in HA titre at higher temperatures was the same for the variants 33 and 196 but the rate of the process by which this increase was mediated seemed to be slower for the variant 196. Virus 23 behaved differently in various

virus stocks For stock B, the increase in HA titre was 16 times and for stock A only 4 times

Effect of Ultrasonic Vibration

In a previous communication (6) it was shown that after ultrasonic treatment of some parainfluenza viruses the HA titre increased 8 to 16 times if the HA test was performed with guinea pig erythrocytes at 4° C Since the variant 196 seemed to produce less haemagglutinin than viruses *23* and *33* (5), the possibility was considered that the low HA titre was due to the presence of 'masked' haemagglutinin which might be released by ultrasonic treatment The viruses were subjected to ultrasonic vibration and the HA titres were determined *before* and after treatment The HA titre increased 8 to 16 times for the variant 196 4 to 8 times for the variant 33 and differently for various stocks of virus 23 The HA titre of one stock (B) increased to the same extent as that of the variant 33, while the HA titre of another stock (A) remained unaltered

TABLE 2

H I Titres Obtained with Untreated and Ultrasonic Treated Virus

Virus	Untreated				Treated			
	HA units	H I titre with antiserum			HA units	H I titre with antiserum		
		23	33	196		23	33	196
23 stock A	4	3200	6400	800	4	3200	3200	800
24 stock B	4	800	800	200	32	800	400	200
					4	3200	3200	800
33	4	1600	1600	400	16	800	400	200
					4	3200	3200	800
196	4	800	800	200	32	400	200	100
					4	3200	3200	800

As a consequence of these results the question arose whether the H I titres obtained with untreated and treated virus would be the same To dilution series of antisera were added 4 HA units of untreated virus, the same amount of virus treated ultrasonically or 4 HA units of treated virus The results in Table 2 demonstrate that the H-I titres were 4 times greater when 4 HA units of treated virus had been used instead of 4 HA units of untreated virus which showed an eightfold increase in HA titre after ultrasonic treatment If the H-I test was performed with virus which showed no increase in HA titre after ultrasonic treatment (23 stock A) the H-I titre was the same for treated and untreated virus

RESULTS

Haemagglutination Spectrum

The ability of the virus 23 and the variants 33 and 196 to agglutinate red blood cells from different species was found to be the same. They agglutinated human group O, bovine, and guinea pig erythrocytes but as a rule not chicken, rabbit, sheep, goat or horse red cells. The HA titres were usually somewhat higher with guinea pig erythrocytes than with human or bovine erythrocytes.

Dependence upon Electrolytes and Influence of pH

Like other myxoviruses (4, 8, 11) parainfluenza 3 virus requires electrolytes for haemagglutination. This was demonstrated in an experiment in which red cells suspended in 0.15 M sucrose solution were added to dilution series of virus in 0.15 M sucrose or in 0.15 M sodium chloride solution.

In order to determine the influence of pH on the HA titer, packed red cells were suspended in appropriate buffers and added to dilution series of virus. Variation of pH in the range pH 5.5 to 9.0 had no significant influence on the HA titer. Similar results have been obtained with influenza virus (9).

TABLE 1
The Influence of Temperature on Haemagglutinating Activity

Virus	4° C	22° C	37° C a)				
	120 b)	120	5	15	30	60	120
23 stock A	16	64	32	64	64	128	64
23 stock B	16	256	128	256	256	256	256
33	32	512	256	256	512	512	512
196	16	128	32	128	128	256	256

a) After incubation at 37° C the titration series were placed at 4° C and read 2 hours after addition of erythrocytes.
b) Time in minutes.

Influence of Temperature

In order to investigate the influence of temperature on the HA titre parallel titrations of the viruses 23, 33 and 196 were incubated with red cells at 4° C and 22° C for 2 hours and at 37° C for various periods. The results recorded in Table 1 show that the HA titre was 4 to 16 times higher after incubation at 22° C and 37° C than after incubation at 4° C. Furthermore the results in Table 1 show that the main effect had been produced within 5 to 15 minutes at 37° C. The increase in HA titre at higher temperatures was the same for the variants 33 and 196 but the rate of the process by which this increase was mediated seemed to be slower for the variant 196. Virus 23 behaved differently in various

eluted at a slower rate than that of the viruses 23 and 33 To exclude the possibility that the observed difference in elution rate depended upon the HA titre of the variant 196 being only one-fourth that of the viruses 23 and 33, another experiment was performed in which the latter viruses were diluted 4 times in Hanks' solution The results (Fig 2) confirmed that the haemagglutinin of the variant 196 eluted more slowly than that of the two other viruses These viruses seemed to have the same elution rate

Besides the ability to agglutinate red cells and to elute, the viruses were found to be able to haemolyse After elution the red cells were nonagglutinable for parainfluenza 3 virus but could nevertheless be agglutinated by fresh red blood cells and by antisera against parainfluenza 3 virus

CONCLUSIONS

As described in a previous paper (5), the variety of cytopathic manifestations which a bovine strain of parainfluenza 3 virus induced in calf kidney tissue cultures was due to the presence of variants in the original population of this strain Two variants were isolated, of which one ('33') had a CPE similar to that of HA 1 virus, while the other ('196') had a CPE similar to that of CA virus The variants were also found to differ with regard to the amounts of haemagglutinin which they produced under the same conditions of cultivation

Further studies on the HA activity showed that the variants 33 and 196 as well as the original virus 23 had the following properties in common ability to haemolyse to form agglutinin on stable cells, and to give a higher HA titre when the titration was performed at 37° C than when it was performed at 4° C Since similar properties have been described for mumps and Newcastle disease viruses (1, 7), these results underline the well-known fact that parainfluenza viruses are more similar to mumps and Newcastle disease viruses than to influenza viruses (2)

As described previously (6) a higher HA titre could also be obtained by ultrasonic treatment of virus The increase of the HA titre by this treatment however was found to vary in different yields of the virus 23 The HA titre of some yields increased to the same extent as that of the variants 33 and 196 while other yields of the virus 23 showed only a minor rise in HA titre or no rise at all after ultrasonic treatment These differences were also reflected in the H I titres Untreated virus for which the HA titre increased after ultrasonic treatment combined with more antihaemagglutinin per HA unit than treated virus or untreated virus with no increase of the HA titre after ultrasonic treatment

The presence of more or less amounts of 'masked haemagglutinin in different virus yields is thus a fact which may affect the H I titre

A clear difference between the variants became evident when the

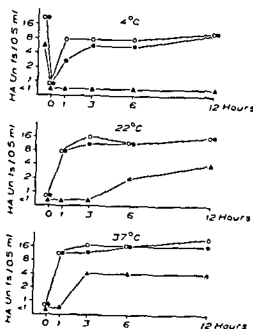


Fig 1

The elution of haemagglutinin at different temperatures

○——○ Virus 23 ●——● Variant 33 ▲——▲ Variant 196

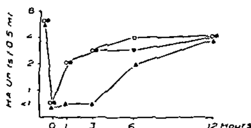


Fig 2

The elution of hemagglutinin at 22° C

○——○ Virus 23 ●——● Variant 33 ▲——▲ Variant 196

Elution Rate

Since the viruses 23 and 33 unlike virus 196 showed a prozone of negative haemagglutination at low temperature it was supposed that the variants differed in elution rate The rate at which the haemagglutinin eluted from guinea pig erythrocytes was investigated in the following experiments

Packed red blood cells were added to the viruses to make a 1 per cent suspension After half an hour at 4° C the red cells were washed twice and resuspended 1 100 in phosphate buffered saline The virus-erythrocyte suspensions were then placed at 4° 22° and 37° C Samples of the suspensions were withdrawn at different intervals centrifuged, and the supernatants tested for haemagglutinating activity The results recorded in Fig 1 show that the haemagglutinin of the virus 196

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THE INFLUENCE OF THYMECTOMY ON ANTIBODY FORMATION

By

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Received 12.viii.60

Extirpation of an organ makes possible an indirect demonstration of its role in antibody formation, *i.e.* serum antibody titres will be lower in the experimental than in sham operated control animals. Experiments of this nature are hardly practical in the case of lymph nodes but the method has been employed with success with spleen and thymus. As long as 1892 *Tizzoni & Cattani* demonstrated lowered titres in splenectomized animals. More recently *Rowley* (1950) found little or no detectable antibody in splenectomized rats after intravenous injection of small amounts of antigen but found antibody formation quite as marked as in normal injected rats after intraperitoneal, intradermal or intraportal injection. Splenectomy experiments have largely confirmed the knowledge derived by other experimental methods of the role of the spleen in antibody formation. In contrast, the influence of thymectomy on antibody formation is not clearly understood and forms the subject discussed in this article.

The influence of thymectomy on antibody formation in rabbits was studied by *Hammar* 1938, *Harris, Rhoads & Stokes* 1948 and *Maclean, Zak, Varco & Good* 1956. Hammar injected S. paratyphi B antigen intravenously 2 months after thymectomy and found slightly lower titres in thymectomized than in control animals. However, the decrease was not statistically significant and the controls were not sham operated. *Harris, Rhoads & Stokes* injected Shigella paradysenteriae or

... and 8 days after thymectomy and ... tween thymectomized and sham ... control animals occasionally fell in a higher group than the thymectomized animals. *Maclean, Zak, Varco & Good* injected bovine plasma albumin intravenously 14 days after thymectomy and found no difference between thymectomized and sham operated rabbits.

In experiments on the specific consequences of thymectomy it is

Supported by the Swedish Medical Research Council.

elution rates of the haemagglutinins were compared The variant 196 eluted slower from the erythrocytes than the viruses 23 and 33 These two viruses showed a rapid elution even at 4° C Thus these viruses also in this respect seemed to be more similar to Newcastle disease virus than influenza virus (11) *Mussgay* (10) has recently described two strains of Newcastle disease virus which, like the variants of parainfluenza 3 virus, differed in elution rate and cytopathogenicity One of these strains eluted slower and was less cytopathogenic in tissue cultures and less pathogenic for chickens than the other strain In our experiments, however, the more slowly eluting variant was equally or more cytopathic than the more rapidly eluting viruses So far nothing is known about the pathogenicity of the variants for calves

SUMMARY

A bovine strain of parainfluenza 3 virus and two variants with different cytopathic effects isolated from this strain were found to have a haemagglutinating activity similar to that of mumps and Newcastle disease viruses *i e* ability to lyse red blood cells, to form agglutinin on stable cells, and to show a higher HA titre when the titration was performed at 37° C than when it was performed at 4° C One of the variants differed from the other variant and from the original virus population by a slower rate at which the haemagglutinin eluted from guinea pig erythrocytes

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Results

The titres of the thymectomized animals tend to be lower than the titres of the sham-operated controls (Table 1)

Experiment 1 b

Since the numbers of animals in experiment 1 a were few, a supplementary group of animals was examined under similar experimental conditions

Methods

19 male guinea pigs weighing 218 268 g at the start of the experiment were used After transport to the laboratory the animals remained in their new cages 7 days before the experiment 9 animals were subjected to subtotal thymectomy and 10 were sham operated according to the schedule of experiment 1 a The animals were otherwise treated exactly as those in experiment 1 a

Results

As in experiment 1 a, the titres of thymectomized animals tend to be lower than the titres of sham operated controls (Table 2)

TABLE 2

Thymectomized guinea pigs	*Sham-operated guinea pigs*
1/256	1/512
1/4	1/256
1/1024	1/2048
1/1024	1/1024
1/64	1/4096
1/1024	1/4096
1/1024	1/512
1/4	1/2048
1/1024	1/4096
	1/4096

Since experiments 1 a and 1 b were performed in the same way, a pooling of their data is justified When this is done, there is a statistically significant difference between thymectomized and sham-operated animals ($0.01 > P > 0.001$, Student's test applied to the inverse of the titre logarithmus)

Experiment 2

This experiment is a repetition of experiments 1 a and 1 b It differs in the following respect the animals were operated upon and given S Typhi H antigen the day after their arrival at the laboratory However, the animals were stressed during the day before the experiment, because they were caught in overly large perforations in the bottoms of their cages

very important to minimize stress. Rabbits are not ideal for thymectomy experiments because of the surgical trauma necessitated by the intra-thoracic location of the thymus. To minimize the disturbing influence of stress the interval between thymectomy and the experiment must be relatively long. However, this course is disadvantageous since functional compensation for thymus loss may occur in the interim period (*Gyllensten* 1953). The guinea pig is the only laboratory animal having a cervical thymus. This animal is thus desirable for thymectomy experiments because at least 95 per cent of the total thymic tissue (*Gyllensten* 1953) may be removed under local anaesthesia with minimal trauma. *Inzerillo & Chili* (1953) performed such subtotal thymectomy on guinea pigs and at an unspecified later time injected Eberthella Typhi intravenously. Only a few animals were examined but these showed lower serum antibody titres in the thymectomized group than in the sham-operated group. The present paper reports similar experiments on the influence of subtotal thymectomy on antibody formation in guinea pigs.

EXPERIMENTAL

Experiment 1 a

This experiment is similar to that of *Inzerillo & Chili* except that antigen was injected immediately before thymectomy. The reason for this procedure will be discussed below.

Methods

19 male guinea pigs weighing 169–223 grams at the start of the experiment were used. After transport to the laboratory the animals remained in their new cages 4 days before the experiment. Subtotal thymectomy was performed on 9 of the animals under local anaesthesia with 1 per cent zylocain (Astra). The 10 control animals were all incized, the thymus mobilized but left *in situ*. The animals were thymectomized or sham operated according to weight with the heaviest animal sham operated, the next heaviest thymectomized, the third in weight sham operated and so forth. Immediately before the operation 0.1 ml per 100 g body weight of a suspension of S. Typhi H antigen (10^9 dead bacteria per cc) was injected into the deep cervical vein. Serum was taken 7 days later and diluted serially. In some series the lowest dilution was 1/5 and others 1/25. An equal amount of S. Typhi H antigen was then added and the tubes incubated in water bath at 37 degrees C overnight. All serum dilutions from the same animal were tested simultaneously with antigen freshly diluted from stock solution. The last tube in the series showing agglutination visible to the naked eye was recorded as the agglutination limit.

TABLE 1

Thymectomized guinea pigs	*Sham operated guinea pigs*
>1/1280	>1/1280
1/160	1/320
1/40	>1/1280
1/1280	1/640
1/320	1/320
1/320	1/1280
<1/10	>1/1280
>1/1280	>1/1280
1/1280	>1/1280
	>1/1280

DISCUSSION

The difference in results of experiment 2 compared to experiments 1 a and 1 b shows the importance of the general condition of the animals in all investigations of thymus function. It is possible that the sham operated animals of experiment 2 were functionally thymectomized by stress with consequent depression of the antibody titres. Unfortunately, no systematic investigations have been published on the influence of stress on antibody formation in young animals. Thymectomy undoubtedly causes greater stress than sham operation, but both are relatively mild traumas. Control experiments cannot be designed without objections of this kind. This objection is less important in view of the fact that thymectomy removes the most sensitive stress target among the lymphatic tissues.

Since thymus is a part of the reticulo-endothelial system (RES) and consequently absorbs some part of the injected particulate antigen, (*Kaplan, Coons & Deane* 1950) the time relation between thymectomy and antigen injection may be important. In the present experiments antigen was injected immediately before thymectomy for the following reason: if antigen is introduced immediately after thymectomy it will theoretically be distributed in a smaller volume of RES tissue than in the case of sham operated animals. The potentially negative effect of thymectomy on antibody formation might then be masked by the higher concentration of antigen in the remainder of the RES tissue. On the other hand, administration of antigen before thymectomy will deprive the animals of part of the injected antigen. It should be emphasized, however, that thymus forms only a relatively small part of the total RES tissue even in small animals. *Juhlin* (1960) showed that less than 1 % of injected small plastic particles were absorbed by thymus RES in young rabbits. This fact tends to reduce the importance of the order in which thymectomy and antigen injection are performed when the interval between the operations is relatively brief.

There was no difference between the titres of thymectomized and sham operated animals during secondary response, indicating that the role played by thymus is relatively small when the entire lymphatic tissue system is challenged by large doses of antigen. However, the results do not exclude an active role for thymus in antibody formation during secondary response.

The thymectomy experiments described here show that thymus very probably plays a part in antibody formation. There is much evidence suggesting that the factor promoting antibody formation is linked to viable cells particularly lymphocytes. This mechanism is reviewed elsewhere (*Fichtelius* 1960).

Methods

19 male guinea pigs weighing 184–246 g were used. Subtotal thymectomy was performed on 10 animals and 9 were sham operated. The animals were otherwise treated exactly as those in experiments 1 a and 1 b.

Results

The titres are lower than in experiments 1 a and 1 b and there is no difference between thymectomized and sham-operated animals (Table 3).

TABLE 3

Thymectomized guinea pigs	*Sham operated guinea pigs*
1/80	1/320
1/640	1/40
1/320	1/160
1/320	1/320
1/1280	1/640
1/160	>1/1280
1/320	1/320
1/640	1/320
1/160	1/160
1/160	

Experiment 3

This experiment compares the influence of thymectomy with sham-operation on antibody production during secondary response.

Methods

18 male guinea pigs were used weighing 152–183 g at the start of the experiment and 257–385 g at the end. Subtotal thymectomy was performed on 9 animals and 9 were sham operated. Primary immunization was carried out on the first day of the expe ... ringe mixture of 0.5 cc agar at 50° C and ... On the 20th day the animals were oper ... given immediately before removal or ... e consisted of 1 cc fluid containing 10^{9} dead bacteria prepared by centrifugation of 3 cc S. Typhi H antigen. Serum was taken 7 days later on the 27th day and the titrations carried out as in the other experiments.

Results

The titres are higher than in the other experiments and there is no difference between thymectomized and sham-operated animals (Table 4).

TABLE 4

Thymectomized guinea pigs	*Sham operated guinea pigs*
1/6400	1/6400
1/6400	1/12800
1/3200	1/3200
1/3200	1/3200
1/800	1/12800
1/12800	1/3200
1/3200	1/1600
1/3200	1/6400
1/3200	1/1200

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MALIGNANT MESENCHYMOMA IN PULMONARY ARTERY AND RIGHT VENTRICLE

Report of a Case with Unusual Location and Histological Picture

By

LARS HAGSTROM

Received 30 viii 60

The pathologist *de Senac* wrote in 1783 "the heart is an organ too noble to be attacked by a primary tumor" This statement opened interest in heart tumors and during the nineteenth century a few reports of such tumors began to appear in the literature However, it was not until 1945 that *Mahaim* carried out the first careful survey on reported heart tumors in his book, "Les Polypes et les Tumeurs du Coeur"

Incidence In Mahaim's work it was shown that primary heart tumors were very rare He found 329 such tumors, 242 of which were benign and of these 49 per cent were myxomas, 25 per cent rhabdomyomas and 26 per cent fibromas angiomas and lipomas together Eighty-seven were malignant The number of malignant tumors has now (1960) increased to about 135 Each year 3–4 new cases are reported

Age and sex Malignant primary heart tumors do not show at any particular age predilection In the literature the age of the cases at death is spread equally from 7 days to 79 years nor is there any clear difference of incidence between the sexes The male-female ratio is 4 5

Pathological Aspects

Macroscopical findings *Whorton* (1948) found that about 2/3 of the malignant primary heart tumors had originated from the base of the heart (atria atrial septum or pulmonary artery), most frequently from the right *atrium* 1/4 *of the* tumors had polypoid projections into the heart chambers some of them thereby blocking the mitral valve in the later part of atrial contraction

Metastases have occurred in about 60 per cent of the cases The most usual location has been *in the lungs* Other organs involved were in order to frequency thoracic lymph nodes, liver, kidney, pericardium adrenals pancreas

Microscopical findings It is difficult to obtain a survey of the pro-

SUMMARY

Thymectomy or sham-operation was made in young guinea pigs immediately after the intravenous administration of S typhi H antigen 7 days later the antibody-titres were lower in the thymectomized animals than in the controls There was no difference between the titres of thymectomized and sham-operated controls during secondary response to the same antigen

The experiments show that thymus very probably plays a role in antibody formation

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Fig 2
Macroscopical picture of the tumor
a Tumor nodule in pulmonary conus
b Tumor masses deforming pulmonary valves
c Firm greyish white lobulated masses in the pulmonary main stem and left main branch
d Yellowish gelatinous tumor masses in the right main and peripheral branches

very troublesome even after slight exertion At the same time the patient had a sudden sharp pain in the region of her left scapula which remained for ten days and which was especially troublesome on deep breathing The patient was again admitted to hospital

During her outpatient observation all laboratory investigations were found to be negative except for a persistent sedimentation rate of 30 40 mm in one hour In hospital the only positive finding was an X ray of her chest in which the left superior lobe showed some scattered "patched and streaked" densities as in TB However th[illegible]
In spite of [illegible]
was carried [illegible]
spring and [illegible]
October wh[illegible]
increased However she had no precordial pain no nocturia nor other signs of cardiac insufficiency The dry cough reappeared but this time it was more troublesome now with a little mucoid sputum occasionally containing small amounts of blood During that autumn she had infrequent sharp pains in the upper part of the back She was again a[illegible]

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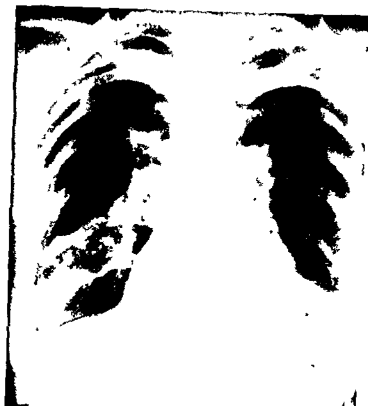


Fig 1
Chest X ray about 6 weeks before death

portion of the different histological types, on account of the large variation in diagnostic criteria and histological terms in use by the various authors

In about 40 per cent of the cases, the tumors have been of the "spindle cell" type, including fibrosarcomas, myxosarcomas, fibromyxosarcomas and leiomyosarcomas, in about 25 per cent they have been of the "round cell" type, including "round cell" sarcomas lymphosarcomas, stem-cell sarcomas and reticulum-cell sarcomas The rest of the cases were diagnosed as angiosarcoma (including Kaposi s disease) giant cell sarcoma pleomorphic sarcoma, embryonic sarcoma mesoblastoma endothelioma and neurosarcoma

REPORT OF A CASE

J J unmarried female housemaid aged 59 (born 1900) (Sabb Hosp Sthlm Clin Rec no 126221/58) came in June 1956 to a hospital in Stockholm complaining of diffuse pains in her abdomen and tiredness She was admitted to the hospital In spite of careful examination, no cause could be found for her stomach trouble However tests on the bicycle ergometer showed that the patient could not even do light work without signs of respiratory and circulatory insufficiency Her abdominal pains improved gradually and she was discharged from hospital and observed However her tiredness remained unchanged until the winter of 1958 when it began to increase slowly She also developed a slight cough Towards the autumn of the same year her earlier moderate dyspnoea greatly increased and after a while was



Fig 2
Macroscopical picture of the tumour
a Tumour nodule in pulmonary conus
b Tumour masses deforming pulmonary valves
c Firm greyish white lobulated masses in the pulmonary main stem and left main branch
d Yellowish gelatinous tumour masses in the right main and peripheral branches

very troublesome even after slight exertion At the same time the patient had a sudden sharp pain in the region of her left scapula which remained for ten days and which was especially troublesome on deep breathing The patient was again admitted to hospital

During her outpatient observation all laboratory investigations were found to be negative except for a persistent sedimentation rate of 30–40 mm in one hour In hospital the only positive finding was an X ray of her chest in which the left upper lobe showed some scattered patched and streaked densities as in TB However the radiologist gave a reserved opinion that metastases might be present In spite of several negative laboratory TB investigations ambulatory TB therapy was carried out during the spring of 1959 The patient felt a little better during the spring and summer of 1959 and worked as a housemaid from 1st April until 5th October when she suddenly fainted in a street At that time her dyspnoea again increased However she had no precordial pain no nocturia nor other signs of cardiac insufficiency The dry cough reappeared but this time it was more troublesome with a little mucoid sputum occasionally containing small amounts of blood During that autumn she had infrequent sharp pains in the upper part of the back She was again admitted to hospital in November 1959 X rays of her chest showed inconstant unspecific small densities around the right hilum and later peripherally in the same lung (Fig 1) During the investigations the patient became weaker and weaker with a picture of respiratory and circulatory insufficiency She died 27 1 60 undiagnosed



Fig 3
Fibrosarcomatous part of the tumor in the pulmonary conus
van Gieson About 400 ×

Autopsy

Macroscopical findings The right ventricle showed a rather marked hypertrophy and the right atrium was markedly dilated The left ventricle was only slightly hypertrophied and there was no dilation of the left atrium

A greyish-white tumor like thickening was found in the upper part of the right ventricle just below the pulmonary valves (Fig 2a) It was about one square centimeter in size and was located in the pulmonary conus towards the septum The *pulmonary valve was partly deformed by a polypoid tumor mass growing out from* the commissure between the posterior and lateral cusps (Fig 2b) The main stem and both main branches of the pulmonary artery were almost completely filled by a lobulated tumor mass, not quite unlike an old organised thrombus Part of the tumor mass was greyish white of firm consistence and lobulated surface while the rest was yellowish soft and rather gelatinous The tumor mass adhered to practically the whole circumference of the main stem and the main branches The greyish white firm mass filled the main stem and the left main branch ending abruptly at the level of the pulmonary hilum presenting a smooth polypoid surface at its end *(Fig 2c) The pulmonary arterial branches distal to this point were free from* obturating growth In the right main branch of the artery the yellowish gelatinous type slightly spotted with red patches dominated (Fig 2d) Only a small part of the tumor in this region had a greyish white colour and firm consistence Only very small passages for the blood stream were left in the pulmonary main stem and the main branches The pulmonary arterial branches of the second and third order in the right lung were to a large extent filled with voluminous yellowish gelatinous tumor masses In some places the tumor masses could be followed in peripheral branches almost to the lung surface The tumor filled arterial branches were highly dilated but no *infiltrative growth into the lung parenchyma could be noted macroscopically*

There was no appreciable blood stasis in the lung parenchyma nor oedema In



F g 4
O e hond o sarc mato s p ctu e in t mor mas es f the pulm nary val e an G e on About 50 ×

I o h lung a number of small mostl recent hemorrhag c nfarcts were found n th per pheral parts more numerous n the r ght than n the left lung There as l lateral y a mo lerate (100 150) pleural effus on w th a sl ght f brinous flocculat on No meta ta es were found n any o gan There were no igns of general blood a is and there ere no patholog cal f nd ng of mportan e n the other organs *M ros oj cal f nd ngs* The tumo like th cken ng in the pulmonary conus sho e l ma nl the p ture f f brous end ca d al th ken ng sharply del neated fr m the m a d m Th
le
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a c ma n wh ch there ha l de eloped small nodules of ver pr m t e ca t lage and al o mall foc w th nd r sp cules f l one (F g 4

The greyi h wh te f rm tum r t ssue n the ma n stem of the pulmonary artery

ue n a n ng mul nu l ated cell F g 5

The yellow h gelat nous tumor n the r ght ma n l ranch f the pulmonar artery ha l the
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trap form and
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nuclear and gi
t ap formed
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The tumor t sue n the per pheral pulmonary arter al l ranches in the r ght lung



Fig. 5

Picture of osteogenous sarcoma in pulmonary left main branch. van Gieson. About 150 ×

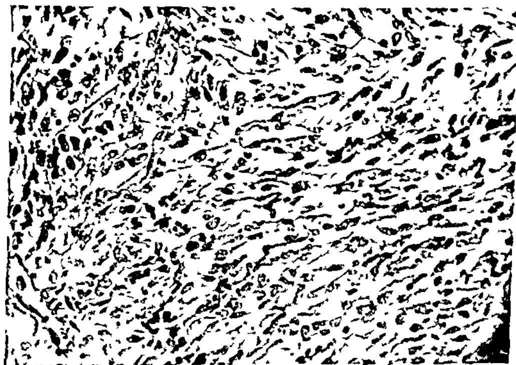


Fig. 6

Rhabdomyosarcomatous picture with tadpole cells in right pulmonary artery branches. van Gieson. About 1600 ×

also showed the histological picture described above In these peripheral branches there were several areas of malignant destruction of the arterial walls and diffuse infiltration of the surrounding lung parenchyma

This tumor was classified as a malignant mesenchymoma with fibrosarcoma osteogenous sarcoma and rhabdomyosarcoma components It is probable that the tumor had arisen in the region of the pulmonary conus and the pulmonary valves The rhabdomyosarcimatous component in the right pulmonary artery system is regarded as a rapidly growing late development of the malignant disease

DISCUSSION

In Table 1 a survey is given of the previously published cases of malignant primary tumors with a similar location as the case described here

TABLE 1

	Year Author	Sex	Age	Prim Location	Metastases	Hist Diagnosis
1923	Mandelstam	M	58	Pulm valves	Lungs	Spindle cell sarcoma
1928	Fschbach	M	58	Pulm artery		Leiomyosarcoma
1928	Frolboese	F	64	Pulm artery	Lungs	Spindle cell sarcoma
1931	Nath	F	50	Pulm artery atrial septum	0	Pleomorphic sarcoma
1934	Kuli b Schuh	M	27	Pulm artery	Lungs left adrenal lymph nodes	Leiomyosarcoma
1 36	G clel	M	47	Pulm artery	0	Pleomorph c sarcoma (Kaposi s disease?)
1939	Mart n Tu hy Will	F	46	Pulm artery	0	Pleomorphic sarcoma
1341	Hawthorn Ray W lff	F	51	Pulm artery	Lungs	Fibromyxosarcoma

The table shows that in none of these cases has the tumor shown a histological picture similar to the case reported here nor does there seem to be any tumor with identical histological picture among the published cases of tumor in other locat on ...
19_6) classified a tumor
mesenchymoma This tum
of fibroblasts collagenous fibres cross striated muscle fibres with a back ground of embryonal mesenchymal tissue This tumor thus seems to be rather similar to the one described here but lacking the component of osteogenic sarcoma

SUMMARY

About 13_ malignant primary tumors of the heart have been described in the literature In most cases they have been located in the base of the heart Some of them have a polypoid projection into the heart cavities but most commonly they have infiltrated the heart walls



Fig. 5
Picture of osteogenous sarcoma in pulmonary left main branch
van Gieson. About 150 ×

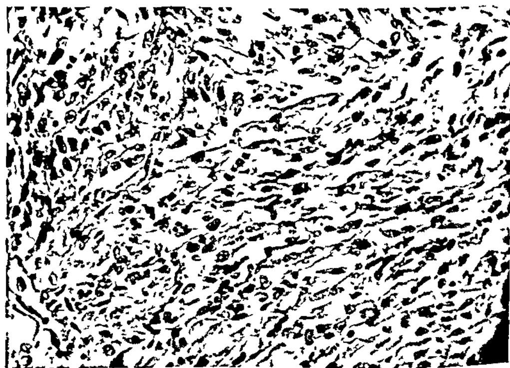


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Rhabdomyosarcomatous picture with tad pole cells in right pulmonary artery branches. van Gieson. About 1600 ×

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DISCUSSION

In Table I a survey is given of the previously published cases of malignant primary tumors with a similar location as the case described here

TABLE 1

Year	Author	Sex	Age	Prim Location	Metastases	Hist Diagnosis
1923	Mandelstam	M	58	Pulm valves	Lungs	Spindle cell sarcoma
1928	Eschbach	M	58	Pulm artery		Leiomyosarcoma
1928	Froboese	F	64	Pulm artery	Lungs	Spindle cell sarcoma
1931	Nath	F	50	Pulm artery atrial septum	0	Pleomorphic sarcoma
1934	Kudlich Schuh	M	27	Pulm artery	Lungs left adrenal lymph nodes	Leiomyosarcoma
1936	Coedel	M	47	Pulm artery	0	Pleomorphic sarcoma (Kaposi s disease?)
1929	Martin Tuohy Wall	F	46	Pulm artery	0	Pleomorphic sarcoma
1941	Haythorn Ray Wolff	F	51	Pulm artery	Lungs	Fibromyxosarcoma

The table shows that in none of these cases has the tumor shown a histological picture similar to the case reported here nor does there seem to be any tumor with identical histological picture among the published cases of tumor in other location in the heart Indeed, *Coulon* (1956) classified a tumor growing in the left ventricle as an embryonal mesenchymoma This tumor arising in a child aged 7 months consisted of fibroblasts collagenous fibres cross striated muscle fibres [illegible]

SUMMARY

About 135 malignant primary tumors of the heart have been described in the literature In most cases they have been located in the base of the heart Some of them have a polypoid projection into the heart cavities but most commonly they have infiltrated the heart walls

The most common histological types have been reported as spindle cell or round cell sarcomas

Here is presented a case of embryonal mesenchymoma (with the components fibrosarcoma osteogenic sarcoma and rhabdomyosarcoma) which arose in the region of the pulmonary artery and which had an extensive outgrowth filling the main stem and main branches of the pulmonary artery It appears that no heart tumor with an identical histological picture has been described previously

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FROM THE HISTOLOGICAL DEPARTMENT, UNIVERSITY OF UPPSALA, SWEDEN
(HEAD M. VRETE M.D.)

THE FREQUENCY DISTRIBUTION OF THE NUMBER AND VOLUME OF THE ISLETS OF LANGERHANS IN MAN

2. *Studies in Diabetes of Adult Onset*

By

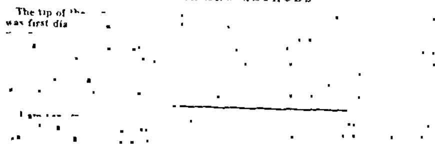
Bo Hellman

Received 30. viii. 60

The arrangement of the islet tissue in non-diabetic humans was, independent of age, found to be characterized by a striking regularity. As regards the actual size distributions of the islets of Langerhans, if the total islet volume was plotted in relation to the islet diameter, the curve obtained was not only symmetrical but for the main part of its course approximated to a normal distribution (*Hellman* 1959 a, b). The mean values and scatter in these distributions were such that a linear relationship was obtained between the logarithms of the number of islets and their diameters in those islet size classes which contained the overwhelming majority of the islets studied (*Hellman* 1959 a, b).

To understand the reactions of the islets of Langerhans in human diabetes, it seems useful to have some knowledge of the actual size distribution of the islets: whether this shows any deviation from the "normal curves" found previously, and if so in what way these results should be interpreted. The present investigation is an attempt to elucidate these questions by studying patients in whom diabetes first became manifest after the age of 45 years.

MATERIAL AND METHODS



The pancreatic specimens were fixed *in Bouin's* solution and after embedding in paraffin were cut into 7 μ thick serial sections. *For the microscopical analyses* sample sections were taken at regular intervals of 308 μ, i.e. every 44th section. After staining with *Gomori's* chrome hematoxylin phloxine method the sections were systematically scanned for islets whose sizes were classified by comparing their projected images at 240 × *magnification* with circles and ellipses of known *areas*. *The relation between the mid class value for the islet diameters in* μ and the *class number has been* given as a nomogram in the caption to Table 1. *From each* pancreas sufficient sections were analysed to bring the number of islet section sur*faces classified to* at least 500. For conversion of the apparent size distribution obtained in this way to *the actual size distribution* the equation system derived by *Wicksell* (1925, 1926) was used. The procedure for this was *the same as that used* in a previous study (*Hellman* 1959 a).

CASE REPORTS

2003. ♂, 71 years old. 5 years ago diabetes was *discovered, which was treated by* dietary restrictions. For 4 weeks before admission to hospital vomiting and abdomi*nal pains*. X ray showed a large cancer of stomach. At subsequent laparotomy this was considered as inoperable and only the tip of the pancreatic tail was resected before the abdomen was closed. Case *No. 1659/59*.

2010. ♀, 68 years old. 2 years ago diabetes was discovered and *treated by* dietary restrictions. For several years pronounced bronchial asthma with cardiac incompensation. Admitted after 2 days' vomiting and severe abdominal pain, and death quickly followed. Autopsy showed a mesenteric gastric thrombosis with gangrene of the small intestine and peritonitis. In the right adrenal gland a walnut sized cortical adenoma was found, in which the cells had the same histological *appearance* as in the normal adrenal cortex. *The* pancreas was resected *20* hours after death. Autopsy No. 32/59.

2012. ♀, 72 years old. 2 years ago diabetes was discovered and was treated by dietary restrictions. Rheumatic fever as a child and later mitral stenosis with [illegible] ... found [illegible] ... orrhage [illegible] ... Autopsy *No. 50/59*.

[illegible] ... n for 10 years. Admitted with acute hemi[illegible] ... ards. Autopsy showed the cause of death [illegible] ... grain sized yellow adenomata were found in the left adrenal gland. The pancreas was resected 16 hours after death. Autopsy No. 82/59.

2020. ♀, 68 years old. 13 years ago diabetes was discovered. It was at first treated *by dietary restrictions* but also during the *past* two years with D 860®. Clinical signs of a nephropathy and retinopathy. Admitted in coma and died rapidly with signs of uremia. The autopsy also revealed gangrenous changes in the distal region of the ileum and bronchopneumonia. The pancreas was resected 30 hours after death. Autopsy No. 629/59.

2022. ♂, 66 years old. 18 years ago diabetes was discovered and treated by diet and also for some months 4 years ago with BZ 55®. At 50 years gastric resection for ulcer. Recently progressive renal insufficiency, with death from uremia. Autopsy revealed the cause of the uremia to be a papillitis necroticans. The pancreas was resected 25 hours after death. Autopsy No. 38/60.

2024. ♂, 74 years old. 11 years ago diabetes was discovered and treated by diet. At 70 years treated *for cardiac infarct. Admitted mentally confused* and died with the picture of circulatory insufficiency. Autopsy showed a severe cardiosclerosis and pronounced acute pyelonephritic changes. The pancreas was resected 16 hours after death. Autopsy No. 138/60.

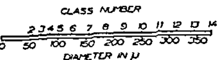
2027. ♂, 70 years old. 3 years ago diabetes was discovered. *It* was at first treated by diet but also during the last six months with D 860®. At the same time as the diabetes was diagnosed a nephrectomy was performed for left sided hydronephrosis and a *hypernephroma* was unexpectedly found in the resected kidney. Admitted febrile and died with renal insufficiency. Autopsy showed pronounced pyelonephritic changes in the remaining right kidney. The pancreas was resected 27 hours after death. Autopsy No. 199/60.

TABLE 1

The Frequency Distribution of the Islet Numbers and Volumes into Different Size Classes Expressed as per 1000

Case no	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	X_{14}
						Islet number							
2003	144 8	146 0	122 3	120 4	180 1	131 5	70 7	41 2	20 9	11 1	0 9	2 3	1 7
2010	436 4	254 2	141 4	86 6	61 4	17 7	2 3		–	–	–	–	–
2012	190 9	193 1	154 2	141 4	170 6	88 9	31 7	13 6	6 3	4 0	3 0	1 4	1 0
2014	322 6	213 2	146 9	119 6	125 4	51 2	16 4	4 6	–	–	–	–	–
2020	315 3	221 8	147 3	99 0	109 4	59 1	28 7	14 1	5 3	–	–	–	–
2022	352 6	234 5	134 4	99 3	101 2	49 3	21 2	6 2	1 3	–	–	–	–
2024	257 4	234 3	144 6	137 4	156 7	49 4	10 6	8 4	1 2	–	–	–	–
2027	247 5	204 9	142 9	121 6	139 9	81 5	38 3	17 0	3 7	2 6	–	–	–
						Islet volume							
2003	7 9	16 9	25 9	42 0	117 4	167 3	155 4	144 0	101 0	82 3	70 0	31 7	30 2
2010	132 0	162 7	165 0	106 9	220 7	124 2	28 4	–	–	–	–	–	–
2012	17 8	39 1	55 6	84 1	189 4	192 6	118 7	80 8	56 2	49 9	52 6	32 8	31 3
2014	56 2	78 7	98 9	132 9	259 0	207 3	115 4	50 7	–		–		–
2020	43 7	65 1	78 9	87 4	180 3	190 1	159 7	124 7	70 1	–	–	–	–
2022	61 7	86 8	90 7	110 7	210 6	200 0	148 7	68 7	22 1	–	–	–	–
2024	41 0	79 0	89 0	139 5	297 2	182 9	67 6	85 0	18 7	–	–	–	–
2027	27 8	48 8	62 0	87 1	187 2	212 7	173 1	122 0	33 3	40 0	–	–	–

The division into size classes is shown in the nomogram, the class numbers are referred to as X_2, X_3 etc. in the text and the Table. It should be noted that the first four classes X_2–X_5 have only half the breadth of the remaining classes.



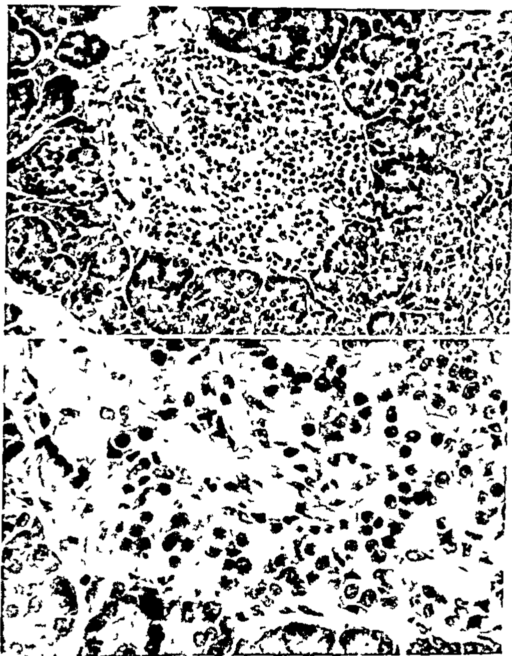


Fig 1

A An example of hyaline changes in an islet frcm case N 2003 The islet is well delimited notwithstanding that more than half f it area is c ccuj ie l l y hyaline deposits × 2?0

B A detail of the lower part f the same islet Between the l yaline lep sits there is real islet parenchyma in the f rm f stran ls With the cl r me l emat xylin phl xine staining used the islet apj ears to c nsist mainly f A cell × 5 0

RESULTS

A qualitative microscopic examination of the islets of Langerhans revealed nothing of especial note in cases 2010 2014, 2020, 2022, 2024 and 2027 In the pancreatic tails of the two remaining cases (2003 and 2012), hyaline changes were observed in many of the islets, in some places being of a rather advanced nature (see Fig 1)

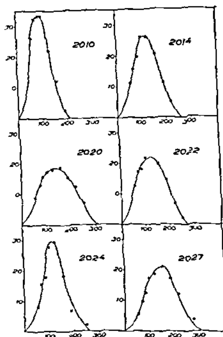


Fig 2

The percentage volume distribution in relation to the islet diameters expressed in μ for cases without hyaline changes in the islets
All the curves show a symmetrical form

The distribution of the islets into different size classes expressed as per 1000 has been given in Table 1 Since the class breadth in the first four size classes [illegible] is only half that of the other classes it is evident that the numerical distribution is in all cases highly asymmetric The number of islets thus increases progressively with decreasing islet diameter While in the majority of the cases the largest islet diameter does not exceed 300 μ, in the two cases with hyaline changes islets were found with a diameter of approximately 400 μ

With knowledge of the numerical size d[illegible]

[illegible] 400) it is evident

that the main part of the total islet volume is contributed by the medium sized islets. If the values are plotted graphically with allowance for the differences in class width, symmetrical curves are obtained for cases 2010, 2014, 2020, 2022, 2024 and 2027 (see Fig 2), which, as a probit analysis reveals, approximate closely to a normal distribution. In case 2003, and particularly in case 2012, the presence of a great many large islets results on the other hand in a definite deviation from the symmetrical form (see Fig 3).

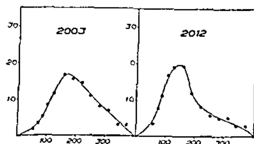


Fig 3

The percentage volume distribution in relation to the islet diameters expressed in μ for the two cases with hyaline changes in the islets. The presence of an excessive high frequency of large islets gives rise to asymmetry in the curves

DISCUSSION

For the calculation of the actual size distribution of the islets the equations given by *Wicksell* (1925, 1926) for the relationship between the observed and the actual size distributions of spherical and ellipsoidal bodies in a section were used. This relationship can be expressed in the form of an *Abelian* integral equation, which after certain approximations can be converted into the *linear equation* system, which is used for the practical calculations. *Hellman* (1959 a) as a result of systematic tests, also showed the validity of using these equations in the studies of the actual size distribution of the islets in the human pancreas. Further, by using only the tip of the pancreatic tail for the analyses, the tendency to a deviation from a symmetrical volume distribution which is sometimes found locally in the fusion area between the ventral and dorsal pancreatic primordia in non-diabetic cases has been avoided completely. It was also found that even after an interval of as much as 27 hours between death and the commencement of fixation, post mortem autolysis did not apparently influence the form of the curves.

It is worthy of note that the islet organ in the majority of cases had retained its regular arrangement in spite of the diabetes occurring in the adult years, i.e. the proportion between large and small islets remained the same as in the control case No 2014 and in other adult non-diabetics (see *Hellman* 1959 a). There are thus no signs that the

diabetes in these cases is associated with a maturity repression of the islets or that there are any regenerative tendencies in the form of an increased number of islets in the smallest size classes

It is of especial interest that in the two cases with hyaline changes in the islets of Langerhans there was a deviation from the symmetrical form of the volume curve manifested as an excess frequency of large islets In assessing the results it must be taken into consideration that the measurements in this case are not representative for the distribution of the real islet parenchyma Since the deposition of the hyaline substance was localized particularly to the large islets which were thus considerably enlarged the form of the distribution curves does not necessarily imply a deviation from the general finding that the islet organ is distributed into different sized bodies according to a simple mathematical relationship

The fact that the volume distribution curves retained their typical symmetrical basic form even in the presence of an adult diabetes can be interpreted as an expression of the inherent effort of the islet organ to retain this balance between the number of large and small islets A similar tendency has been shown for example in the rat (*Hellman* 1959 c) In this animal in spite of the changes in the total volume and composition of the islet organ which were observed with either of two extreme diets and also after protracted administration of small doses of alloxan the volume distribution of the islets still remained regular It may also be pointed out that the volumetric distribution curve is still symmetrical in the recessive obese hyperglycemic syndrome in mice in spite of a nearly ten fold increase in the islet volume (*Hellman Brolin Hellerström & Hellman* 1960)

SUMMARY

The actual size distribution of the islets of Langerhans was studied in seven adults with diabetes in whom the disease first became m

previously for non diabetics In the two remaining cases the volume distribution curve was however asymmetric because of an excessively high frequency of large islets This does not necessarily imply any deviation from the general finding that the "real islet parenchyma is distributed into different sized bodies according to a simple mathematical relationship since there was in these cases also a deposit of hyaline substance localized particularly to the large islets

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FROM THE INSTITUTE OF PATHOLOGY AND THE DEPARTMENT OF THORACIC SURGERY UNIVERSITY OF LUND SWEDEN

FIBROXANTHOMA OF THE LUNG WITH BRONCHIAL INVOLVEMENT

By

ULLA ISING M D and ERLAND LINDER M D

Received 7 x 60

Benign lung tumors and bronchial tumors are fairly rare—apart from bronchial adenoma— but with increasing diagnostic and surgical possibilities classification of these tumors is becoming more important The most common benign mesenchymal tumors of the lung are fibroma chondroma and lipoma usually arising from the larger bronchi after which come leiomyoma lymphoma and myoblastoma Angiomas and hamartomas occur particularly in peripheral portions of the lungs The literature contains reports of only a few cases of *fibroxanthoma* (Table) to which three personal cases may now be added

REPORT OF CASES

Case 1 After a feeling of tension in the precordium for about a month and a cough for about a week a surgeon *aged 28 who had hitherto felt well* coughed up a pea sized smooth surfaced semi sol d mass and about 100 ml of blood Some days

certainty (Fig 1) Scattered vessels were visible in the tissue which was however not particularly vascular Minute amounts of iron demonstrated by the Turnbull method were found in a few of the spindle shaped cells as well

suggesting an inflammatory irritation The picture was similar to that of a fibroma rich in cells *Diagnosis* Fibroxanthoma No histologic signs of malignancy

TABLI'

Author	Age of patient	Site of tumor	Connection with bronchus	Symptoms	Other diseases	Histologic picture
Csermely	37♂	Lob sup dxt	Whole lobe involved	–	7 yrs. earlier operated for benign fibroma, same site	*Benign*
Lingerland	45♀	Lob inf sin	+	Hemoptysis	Tuberculous cavity near tumor	Benign
Lingerland	20♀	Lob med dxt	+	Cough	–	Benign
Essunkin	48♀	Lob inf dxt	+	Bronchitis		Malignant
Scott Morrow Payne	34♀	Lob inf dxt	?	Hemoptysis Pleurisy	–	Benign
Umiker Iverson	24♂	Lob sup dxt	?	Cough	–	*Benign*
Umiker Iverson	35♀	Pulm sin	?	Bronchitis	–	Benign
Umiker Iverson	41♀	Pulm dxt	+	Bronchitis	–	Benign
Author's case 1	28♂	Left main bronchus	+	Opressive chest tension	–	Benign
Author's case 2	24♀	Lob inf dxt	+	–	—	Benign
Author's case 3	15♂	Lob inf sin	+	–	—	Benign



Fig 1

Case 1 Base of tumor fragment coughed up Bands of spindle shaped cells with slight polymorphism Few mitoses Groups of xanthoma cells Eosin haematoxylin × 140

The patient has felt well since the operation 12 years ago Chest X ray studies have revealed no abnormalities The patient shows no evidence of xanthomatous changes in other organs and no cutaneous xanthomas Determination of the blood lipid content was not performed

Case 2 On routine mass chest X ray examination a round parenchymal density was found in the periphery of the right lower lobe of a 24 year old woman Roentgen examination on admission (Fig 2) showed that the tumor was situated in the anterobasal segment of the right lower lobe close to the oblique fissure Neither ordinary roentgenography nor body section roentgenography revealed any calcifications She had no symptoms of lower respiratory tract disease and there were no abnormal physical and laboratory findings

Thoracotomy was performed No pleural adhesions were seen In the anterobasal segment of the right lower lobe, close to the fissure, the lung surface was slightly violet In this region a plum-sized delimited tumor was palpated in the parenchyma During palpation the violet discoloration became more intense and the tumor became softer On incision the cut surface of the tumor was found to be brittle It was broadly and intimately connected with a sub segmental bronchus The growth was radically removed by a selective sub segmental resection The postoperative course was uneventful and on section 19 — ...

... but no cell polymorphism In

fibrils ...

also the ...

in palisa ... few vessels were seen in the tissue Part of the tumor



Fig 2
Case 2 Antero posterior view An almond sized tumor is seen laterally at level of ninth rib on right side

was enclosed by a thin capsule of connective tissue containing a few wide vascular spaces There were no signs of infiltration into surrounding lung tissue In some regions a few round cells were detected in the tissue Nowhere was the tumor lined with epithelium but it was situated in the peribronchial connective tissue of a large bronchus and some wide vessels were seen in the tumor capsule

Diagnosis Fibroxanthoma

Case 3 A 15 year old boy who at 11 years had fractured his nose had otherwise always felt well On mass chest X ray examination a plum sized round tumor was discovered in the left lower lobe Reexamination of a massradiographic film taken 8 years previously showed no evidence of a pathologic condition (Fig 4) Roentgen examination of the chest on admission to the hospital revealed a round mass about 4 cm in diameter and situated centrally in the left lower lobe with a wedge shaped density extending towards the basal surface of the lobe Bronchography showed that the tumor compressed the bronchus of the lower lobe at the level of the hilus of the basal segments and thereby almost completely obstructed the further passage of the contrast medium The basal segments were atelectatic The roentgen appearance was the same as that found one month previously

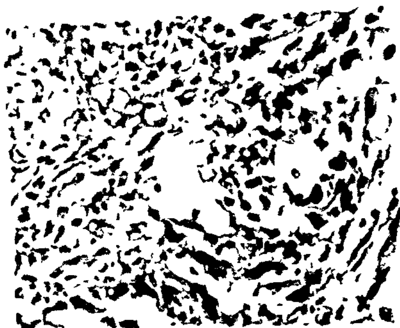


Fig 3

Case 2 Loose groups of spindle shaped or slightly polymorphic cells with slight nuclear polymorphism Small groups of large pale xanthoma cells Eosin haematoxylin × 480

With the exception of the roentgenogram no abnormal physical or laboratory data were found

At left sided thoracotomy no pleural changes were found A fairly soft rounded tumor the size of a mandarin was felt in the center of the lower lobe It did not extend down to the visceral pleural surface From the oblique fissure an incision was made through a small parenchymal bridge to the center of the brittle and disintegrating tumor into which emptied a basal segmental bronchus The unfavorable site of the tumor made it necessary to excise the lower lobe The post operative course was uneventful Six months after the operation the patient had no symptoms and felt well

On macroscopic examination part of the surface of the tumor was found to be smooth while other parts were not well defined against the contiguous lung parenchyma The tumor was firmly adherent to and embraced part of a large bronchus of the lower lobe It was elastic and the cut surface was slightly granular dark brown red with irregularly shaped yellow brittle parts

Histologic examination showed the tumor to be composed of criss cross bands of spindle shaped cells with oval nuclei moderately rich in chromatin Some parts [illegible]

shaped cells contained no fat Abundant iron pigment was often seen in the xanthoma cells as well as in some of the spindle shaped cells Some vessels ran through the tissue Occasional round cells were noted Within one region the tumor was adjacent to a bronchus and was closely connected with the bronchial cartilage The sections examined showed no direct contact between the tumor and the bronchial epithelium

Diagnosis: Fibroxanthoma

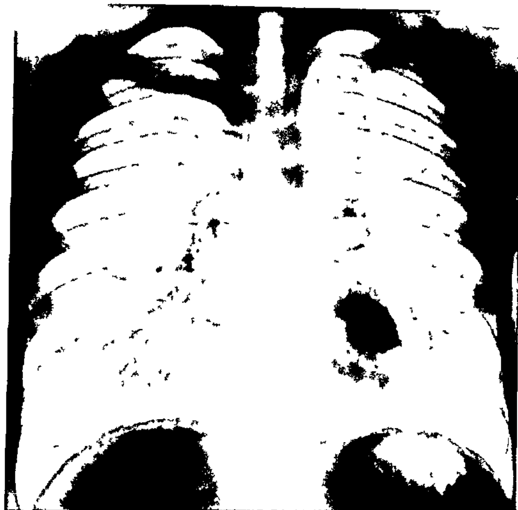


Fig 3
Case 3 Antero posterior view Plum sized well defined tumor in left cardiac outline and partly overlapping heart shadow

DISCUSSION

Tumor-like formations of the same histologic appearance as in the three cases described above are not uncommon in the subcutaneous tissue Several similar formations situated in the mediastinum (2 4 8) or retroperitoneum (12, 18) have been described as well as a few originating from the periosteum (1 13 19) and stomach (16) The literature contains reports of eight cases of lung tumor of the same histologic appearance (Table) These tumors are built up mainly of two different types of cells, namely spindle-shaped, fibroblast like cells, and xanthoma cells containing fats some of which are double refractive

The nature and origin of these formations are still obscure Whether they are the result of an inflammatory-granulomatous process a manifestation of a generalised or a local storage disease or a true neo-



Fig 5

Case 3 Groups of xanthoma cells and bands of spindle shaped cells No cellular polymorphism and no mitoses A few round cells are seen between the tumor cells Eosin haematoxylin × 210

plasm is not entirely clear These possibilities were discussed as early as 1927 by Brandt in his description of a fibroxanthoma of the posterior mediastinum He considered the possibility of a storage disease less likely *Oberling* (12) described three cases of retroperitoneal fibroxanthoma which he believed to be of inflammatory origin The theory of the inflammatory origin has been supported by *Czermely* (3) and by *Gruenfield et al* (7), who stressed the similarity to the xanthomatous granulation tissue seen around colonies of actinomyces They also emphasized that one often sees typical lipoid-containing foam cells in salpingitis, in the walls of abscesses, in lepragranuloma in scar tissue etc The possibility of actinomycosis in the three cases described in the present paper may be excluded In one of the cases of fibroxanthoma of the lung described by *Fingerland* (6) a tuberculous cavity was found close to the tumor but this was probably just a coincidence *Umiker & Iversen* (17) interpreted their four cases of lung fibroxanthoma as "post inflammatory tumors" In all of them the patient had had an infection of the respiratory tract before the discovery of the tumor but these infections might very well have been secondary to the tumors

Most of the cases of fibroxanthoma of the lung in the literature showed more or less abundant inflammatory cells and in our three

cases there was also a sparse accumulation of round cells and polymorphonuclear leukocytes between the fibroblast-like cells The occurrence of these cells does not necessarily mean that the process was of inflammatory origin, because they might represent a secondary inflamation None of the three patients described had symptoms of respiratory tract infection, and at operation the lungs showed no signs of a pathologic condition The fact that fibroxanthoma is very rare despite the high incidence of respiratory tract infections also argues against the growth being of inflammatory origin Lipoid pneumonia shows an anatomic picture differing entirely from that of fibroxanthoma

Thanhauser et al (15) and *Gruenfield et al* (7) have thoroughly discussed the possibilities of fibroxanthoma being a manifestation of a local storage disease or a part of a generalised storage disease In no case of fibroxanthoma of the lung has the blood cholesterol or total fat been determined and in the case of fibroxanthoma that was located in the stomach the blood cholesterol was found to be normal (16) In the three present cases the blood lipids were not determined, but the patients had no signs of hypercholesterolaemia or of lipid storage elsewhere

There is much to suggest that the xanthomatous formations are true tumors Of the cases on record, only one showed a sarcoma-like histologic picture (11), and in none were any metastases seen There were no signs of malignancy seen in the present cases The formations thus appear to be benign

There has been some debate as to whether they represent a special type of tumor or whether they should be regarded as some form of fibroma, myoma neurinoma or angioma *Liebow & Hubbel* (10) described seven cases and *Essbach* (5) one case of angiomatous lung tumor with segments whose histologic structure was similar to that found in the present cases of fibroxanthoma It is therefore possible that the fibroxanthoma might be a type of sclerosing angioma of the lung In our case No 1 the patient lost a fair amount of blood (about 50 ml) immediately after he had coughed up a fragment of the tumor which suggests that the latter was fairly vascular In case No 2 the tumor bled profusely at operation and histologic examination revealed a number of wide vascular spaces in the periphery

Our case No 1 as well as some of the cases described in the literature showed small parts histologically identical with the picture of a fibroma without any demonstrable fat in the cytoplasm of the cells In addition, a small amount of fine collagen fibrils is often seen in fibroxanthomas The possibility of the tumor being a degenerative fibroma must therefore also be considered *Finally it is possible that these* tumors should be regarded as mesenchymal tumors with simultaneous differentiation of fibroblast-like cells as well as histiocyte elements with phagocyting capacity

The genesis and nature of fibroxanthoma must still be regarded as obscure It is possible that a fibroxanthoma might sometimes develop from a tumor that was primarily an angioma but in other cases from a primary fibroma

In most cases of fibroxanthoma of the lung (Table) the tumor was connected with some bronchus and probably the tumor was primarily localized to the bronchus This assumption is also supported by the observation that the tumor often causes bronchial symptoms such as coughing bronchitis and hemoptysis and in our case No 1 the tumor was situated entirely intrabronchially and the major part of it was coughed up Also in those cases where the tumor had spread peri bronchially it probably originated from some bronchial structure

Irrespective of their origin these tumors are of interest from a clinical differential diagnostic point of view The roentgenographic appearance is not characteristic and the tumor can therefore not be distinguished from malignant or non malignant lung tumors or from tumor like inflammatory conditions Their gross appearance at opera tion may also be diagnostically misleading because the xanthomatous regions involved are brittle yellow white granular and to some extent resemble tuberculous caseation

The tumors described (see table) occurred in both sexes mainly in young people and they appear to be non malignant

SUMMARY

Three cases are described of fibroxanthoma of the lung one situated intrabronchially The other two were connected with a bronchus The few cases hitherto published are given in tabular form The origin and nature of the formations is discussed and their clinical characteristics are described

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PROBABLY NEOPLASTIC PROLIFERATION OF LYMPHOID TISSUE (FOLLICULAR LYMPHO-RETICULOMA)

Reports of Four Cases with a Survey of Literature

By

LENNART ZETTERGREN

Received 7 x 60

Over a period of seven years, I have had the opportunity to observe a total of four cases of a no doubt rare and, from pathological points of view, characteristic lesion taking the form of tumour-like proliferation of lymphoid tissue Since this lesion has not been described hitherto in the Scandinavian literature, and as my opinions about it are at variance with those expressed by previous workers, I deem the publication of this paper justified

CASE REPORTS

Case 1 O P a male aged 2[illegible] … a lump growing in the left … good general health with an … swelling of the lymph nodes … was about as big as a wal[illegible] … underlying tissues An attempt to extirpate it met with no difficulty During an observation period of about 7 years there has been no recurrence, his general health has been good and he is in no way hampered in his everyday activities

Case 2 T J a male aged 45 Previously he had always been in good health In 1956 he consulted a physician for a tumour which he had become aware of about a month previously in the right supraclavicular fossa It had recently seemed to grow larger and he had felt [illegible] … [illegible] cytes and 10 per cent monocytes A follow up examination a year later disclosed [illegible] … There has been no recurrence during a [illegible] …

Case [illegible] … she consulted a physician for a tumour … years The tumour had grown slowly … caused the patient any distress in the form of pain or tenderness However she complained of the fact that recently her right arm had tended to tire more easily than the left At examination [illegible] … enlarged At operation it

was found that the resistance was not an authentic mammary tumour but a growth located in the pectoral muscle It was covered by a thin layer of muscular tissue and was easy to enucleate, except at the base which was attached to the underlying tissues by ly be done by means of ligature ule might not have been remo Three months after the intervention a fusiform, non tender resistance a bit thinner than a pencil was palpable in the pectoral muscle beneath the operative scar Just under a year later the resistance had diminished in size No signs of recurrence were observed when the patient was followed up two years subsequently

Case 4 F R, a registered nurse aged 30 From 1929 to 1945 she had served as a missionary in the Congo where she had undergone three attacks of fever interpreted as malaria A routine fluoroscopic examination in the end of April 1958 disclosed the presence of a mediastinal tumour A more complete examination in June the same year revealed width increase at hilar level of the anterior portion of the mediastinum, the region being the site of a plum sized soft-tissue density which was confluent with the anterior portion of the mediastinum and sharply demarcated from adjacent pulmonary parenchyma Tomographs disclosed that the density was situated anterior to and laterally of the aortic arch and seemed to extend medially behind the sternum Angiographs showed that the tumour in no way directly involved the heart or the large vessels The patient's general health was good and the superficial lymph nodes were not enlarged The spleen was not palpable The blood was normal The E S R was 12 mm in one hour The patient was operated upon and it appeared that the tumour was as big as a hen's egg, faintly lobated and well encapsulated It had a homogeneous and lardaceous cut surface The postoperative course was satisfactory

PATHOLOGICAL OBSERVATIONS

Grossly as well as microscopically the four tumours—three extrathoracic and one intrathoracic—were similar in appearance They had a firm consistency, were well circumscribed, had a thin fibrous capsule, and exhibited a faintly lobulated, greyish-yellow, lardaceous, glistening cut surface Microscopically the tumours were composed of a lympho-reticular tissue rich in cells, predominantly lymphocytes The tissue exhibited a follicular patterning (Figs 1 and 2) with groups of lymphocytes of practically the same size fairly uniformly distributed over the surface of the section These lymphocytes were often disposed in concentric rings about the midpoints of the follicular foci which were the site of small rounded clusters of large, pallidly plasmatic reticular cells having round, vacuolized nuclei (Fig 3) In most places the arrangement of these cells provided at least a faint impression of concentric layering These cell clusters were not too unlike germinative centres (Fig 4) and some of them were also rather like Hassall's corpuscles (Fig 3) A disordered mixture of lymphocytes and reticulocytes was present between these follicular foci So was a fairly large amount of collagenous connective tissue which was partially affected by hyalinization (Figs 2 and 5) The lymphocytes between the hyaline connective tissue trabeculae were disposed in rows, an arrangement imparting to the tissue an appearance resembling that of certain angiomatous tumours (Fig 5) The lympho-reticular tissue was highly vascularized and the vascular endothelium hyperplastic Especially the larger vessels exhibited thick coatings of a partly laminar, hyalinized connective

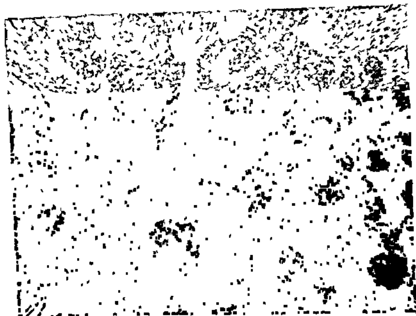


Fig 1
(Case 1) General view showing follicular structure of lesion
Haematoxylin van Gieson

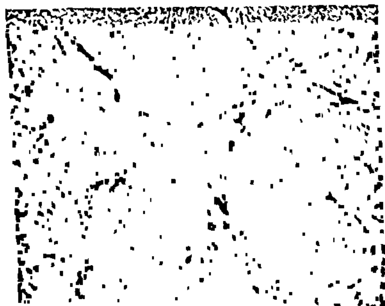


Fig 2
(Case 2) Closely spaced follicular foci mainly composed of lymphocytes in a lympho-reticular tissue with finely fibrillar, hyalinized collagen connective tissue Silver impregnation according to Wilder

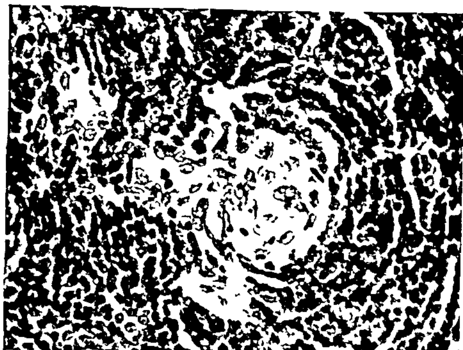


Fig 3
(Case 4) Centre of a follicular focus with a small group of reticular cells vaguely resembling a Hassall's corpuscle Haematoxylin-van Gieson

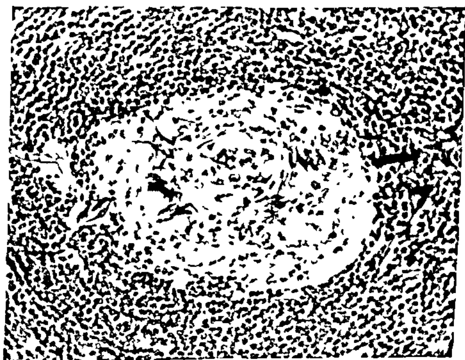


Fig 4
(Case 4) Centre of a follicular focus with a larger group of reticular cells Haematoxylin van Gieson



Fig 5
(Case 2) Detail of the lymphoreticular tissue between the follicular foci Note the arrangement of the lymphocytes between the trabeculae of hyaline tissue Haematoxylin van Gieson

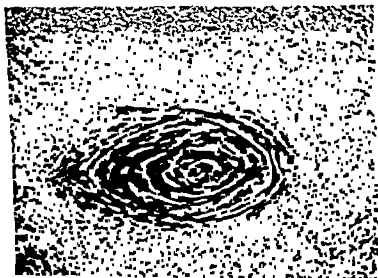


Fig 6
(Case 4) Whorl like perivascular hyalinosis Haematoxylin van Gieson

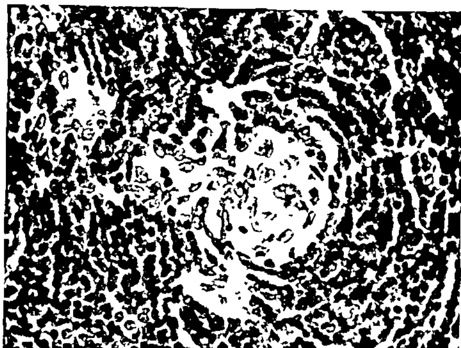


Fig 3
(Case 4) Centre of a follicular focus with a small group of reticular cells vaguely resembling a Hassall's corpuscle Haematoxylin van Gieson

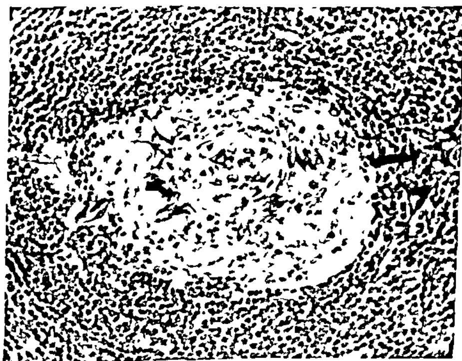


Fig 4
(Case 4) Centre of a follicular focus with a larger group of reticular cells Haematoxylin van Gieson



Fig 5

(Case 2) Detail of the lymphoreticular tissue between the foll cular foci Note the arrangement of the lymphocytes between the trabeculae of hyaline tissue Haematoxylin van Gieson

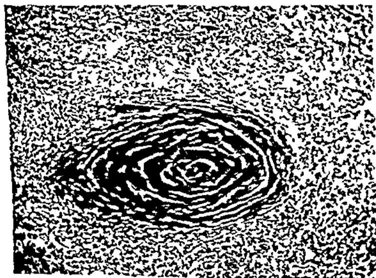


Fig 6

(Case 4) Wh rl l ke perivascular hyalin sis Haemat xylin van Gies n



Fig. 7

(Case 2) A follicular lymphocyte focus with peculiar ramifying vessels. Silver impregnation according to Wilder.



Fig. 8

(Case 3) Sharp demarcation between neoplastic tissue and the pectoral muscle. Haematoxylin van Gieson.

tissue (Fig 6) But perivascular hyalinosis was also encountered around the small vessels, particularly around those vessels which entered and in a characteristic manner ramified within the central portions of the follicular foci (Fig 7) Mitoses were rarely seen Grossly as well as microscopically the tumours were invested by a thin capsular connective tissue envelope which, however, here and there was absent or infiltrated by lymphocytes In one of the cases (Case 3) the adipose tissue adjacent to the tumour presented a localized region which was infiltrated by lympho reticular tissue On the other hand, the tumour was sharply demarcated from adjacent portions of striated muscular tissue (the pectoral muscle) (Fig 8) Neither sinusoidal patterning nor genuine Hassall s corpuscles were observed in any of the tumours In Case 3, a small lymph node of normal appearance was contiguous with the tumour It was separated from the tumour merely by thin bands of collagenous connective tissue In Case 4, too, unchanged lymph nodes were present in the immediate vicinity of the tumour No plasma cells were encountered in the lymphoreticular tissue

PREVIOUSLY REPORTED CASES

So far as I have been able to determine, 19 cases of this lesion are on record (Table 1) In 1953 *Crane & Carrigan* published the first case under the title "Primary Subpleural Intrapulmonic Thymoma" The case occurred in a female aged 19 who had been admitted to hospital in order to establish the cause of chronic asthenia Examination disclosed enlargement of the axillary lymph nodes on both sides X-ray examination of the chest revealed the presence to the left of the aortic arch of a distinctly circumscribed density measuring about 3 cm by 4 cm which moved in unison with the pulsations of the aorta At operation it was found that the firm tumour was situated beneath the mediastinal and visceral pleurae next to the left pulmonary radix where, moreover, numerous soft, anthracotic lymph nodes also were seen Microscopic examination of the tumour disclosed that it was composed of lymphocytoid cells disposed in a follicular pattern Large, acidophilic, reticular cells were encountered between the follicular foci Similar cells were also observed at the middle of the follicles In addition the tissue contained some large, concentrically layered, fibrohyaline structures resembling Hassall s corpuscles The lymph nodes exhibited no abnormalities apart from anthracosis Remarkably enough the patient s prolonged asthenia improved postoperatively

Castleman (1954) reported the second of these cases at a Staff Meeting at the Massachusetts General Hospital, Boston, Mass The patient was a male aged 40 who had been appendectomized 13 years previously owing to repeated attacks of abdominal pain combined with hyperpyrexia Nine months after the operation he had yet another febrile attack, and a roentgenographic examination of the abdomen was car-

TABLE 1

enty Three Cases of Probably Neoplastic Proliferation of Lymphoid Tissue

Case No	Author	Age & sex	Initial Complaint	Tumour Duration yrs	Size cm	Location	Follow-up yrs
1	Crane & Carrigan 1953	19 F	Fatigue	–	4 3 × 3 5 × 2 cm	Root of left lung beneath pleura connected with apical superior bronchus, L U L	2
2	Forse et al 1953	45 M	X ray finding	1	6 × 5 cm dia (70 gm)	Close to trachea and superior to right main stem bronchus	–
3	Castleman 1954	40 M	Chronic cough	8	9 × 11 × 4 cm	Left anterior mediastinum over left pulmonary artery and close to phrenic nerve	3
4	Castleman et al 1956	30 M	X ray finding	6	3 5 cm dia	Subpleural at right hilum adherent to superior segment bronchus	2
5	Ibid 1956	30 F	"Colds"	4	12 × 3 × 2 cm	Between superior vena cava and trachea	5
6	Ibid 1956	25 F	Cough	–	6 × 4 × 3 cm	Left hilum at interlobar fissure	2
7	Ibid 1956	44 F	X-ray finding	5	5 × 5 × 3 5 cm	Posterior mediastinum extending along right bronchus and lower trachea	½
8	Ibid 1956	54 M	X ray finding	1	6 cm dia	Adherent to under surface of upper third of sternum, bordering innominate vein	1
9	Ibid 1956	20 M	Retrosternal ache	2	5 × 4 × 3 5 cm	Superior and posterior to left main stem bronchus on main pulmonary artery adherent to pericardium and parietal pleura	1

10	Ibid 1956	23 F	X ray finding	6	7 × 5 × 3 cm (100 gm)	Right anterior mediastinum with extension into supraclavicular fossa	—
11	Ibid 1956	22 M	X ray finding	—	8 × 5 × 3 cm (67 gm)	Superior anterior mediastinum	—
12	Ibid 1956	24 M	X ray finding	—	15 × 11.5 × 6 cm	Right anterior mediastinum	1
13	Ibid 1956	22 M	X ray finding	½	5 × 4 × 3 cm	'Mediastinal mass'	1
14	Cohen 1957	14 F	Pain and tenderness	2½	5.5 × 4 × 3 cm	Deltoid region of the left arm	3
15	Ibid 1957	58 F	Pain and discomfort in the chest Cough	½	8 × 6 × 4 cm (150 gm)	Posterior superior mediastinum between trachea and oesophagus	2½
16	Inada & Hamazaki 1958	18 M	Fatigue X ray finding	2	0.6 × 1.1 × 0.7 cm	Left hilum closely related to the left pulmonary artery and to the aorta medially	—
17	Kurebane et al 1957	14 F	X ray finding		Hen's egg	Right hilum interlobar	—
18	Wada et al 1957	24 F	'Colds'	—	Apple	Right anterior inferior mediastinum	—
19	Nakano & Shimomura 1958	30 F	X ray finding	5	Two tumours—one small fist, one walnut	Left hilum	—
20	Zettergren 1960	27 M	No	2	Walnut	Left axilla	7
21	Ibid 1960	45 M	Pain	½	Hen's egg	Right supraclavicular fossa	3
22	Ibid 1960	23 F	Tiredness of right arm	5 6	Hen's egg	Between left breast and pectoral muscle	3
23	Ibid 1960	30 F	Febrile attacks	¾	Hen's egg	Left anterior mediastinum	2

ried out Considering that it disclosed the presence of a calcified deposit, tuberculous mesenterial lymphadenitis was considered a probable diagnosis On the same occasion roentgenograms of the chest showed no abnormalities A routine x-ray examination of the lungs done four years later disclosed the presence of an intrathoracic tumour next to the aortic arch Approximately eight years later the patient was admitted to hospital for hyperpyrexia, leukocytosis, anemia and an accelerated E S R The intrathoracic tumour was still present and, as it was believed responsible for the symptoms, the patient was operated upon It was then found that the large tumour, measuring 9 cm by 11 cm by 4 cm, was located to the left of the medial line in close proximity to the left main branch of the pulmonary artery and the heart A large number of enlarged lymph nodes were encountered adjacent to the tumour The tumour had a fairly soft consistency and a greyish-pink cut surface *Microscopically it was composed of hyperplastic lymphoid* tissue with numerous germinative centres surrounded by mature lymphocytes A high proportion of the germinative centres exhibited hyalinized foci *Castleman* expressed the opinion that the neoplasm possessed no genetic relationship to the thymus He claimed that he had analyzed all cases of thymoma and of other mediastinal tumours in the records of the Armed Forces' Institute of Pathology and discovered four similar cases He said he had shown the sections to several pathologists specializing in lymphadenopathies all of whom agreed with his view that it was not a thymoma He thought there was no risk of recurrence It was stated in the ensuing discussion that the cases observed by *Castleman* probably represented a new disease entity In the "Atlas of Tumour Pathology", *Castleman* (1955) described the five cases he had assembled under the title "Hyperplasia of Mediastinal Lymph Nodes"

In a subsequent paper entitled "Localized Mediastinal Lymph-Node Hyperplasia Resembling Thymoma", *Castleman, Iverson & Menendez* (1956) provided an excellent survey of the clinical and pathological features of the lesion Their observations were based on no fewer than 13 cases gathered from a variety of U S Clinics and Departments of Pathology Two of these cases had been published previously, *viz* the aforementioned case reported by *Crane & Carrigan* (1953) and another case published under the title "Ectopia of Primary Thymic Tumours" by *Forsee, Farinacci & Blake* (1953) The majority of the patients displayed no symptoms of their condition which was manifested at routine X-ray examinations of the chest Some of them had consulted a physician for upper respiratory tract infections and coughing Definite signs of myasthenia gravis were in no case seen The benign character of the lesion was evidenced by the facts that in a number of cases it had remained roentgenologically stationary for upto eight years and that no case of recurrence was known *In the large majority of cases the* mediastinally located tumour was about as big as a hen's egg Two

dominant features characterized the microscopical appearance of the

a thymoma On the other hand, the view previously expressed by *Castleman* that it probably was a matter of inflammatory lymph-node hyperplasia was reasserted Among the facts *Castleman et al* marshalled in support of this view, the abundance of interfollicular plasma cells encountered in most of the cases was one

Cohen (1957) reported two additional cases of this type under the designation "Tumor Like Proliferations of Lymphoid Tissue" One of them occurred in a girl of 14 with a tumour in the deltoid region for the past 2½ years The well demarcated tumour weighed 33 g, was firm and had a cut surface which was light brown with white streaks The other case involved a man aged 58 years complaining of progressive fatigue, exercise dyspnea and coughing X-ray examination of the chest disclosed the presence of a mediastinal tumour At operation it was found in the postero cranial part of the mediastinum between the tra-

as well circumscribed and had the microscopical appearance that described by *Castleman et al* No histological signs of malignancy were seen, and the patients remained free from symptoms throughout the postoperative observation period of 3 and 2½ years respectively

In a communication entitled "Localized Mediastinal Lymph-Node Hyperplasia", *Inada & Hamazaki* (1958) recently reported a case belonging to the same category It occurred in a man of 18 as a tumour the size of a hen's egg in the left hilar region The growth was partly covered by the visceral pleura and adhered firmly to the apico-posterior segment of the superior lobe It was closely related to the left pulmonary artery anteriorly and to the aorta medially The tumour was circumscribed by a thin capsule The freshly cut surface was greyish-pink and parenchymatous Its microscopical picture was the same as that of the cases reported in the foregoing The fact that *Inada & Hamazaki* noted altered vessels with central whorling resembling Hassall's corpuscles is interesting So is their observation that the lymphoid tissue extended into the adjacent lung parenchyma A small lymph node encountered near the main mass exhibited the picture of lymphadenitis simplex With respect to the nature of the lesion, *Inada & Hamazaki* emphasized that the infiltration of the lung parenchyma favoured a neoplastic process rather than simple hyperplasia due to chronic inflammation Moreover, if chronic inflammation were the sole causative agent, they found it hard to conceive, why these characteristic changes should occur almost exclusively in the mediastinal lymph nodes and in most cases involve only a single lymph node

In 1959 *Inada et al.* reported yet another case of the same kind. The patient was a woman of 54 who for some months had complained of fatigue and back pain. The well encapsulated tumour was situated in the superior part of the mediastinum and weighed nearly 400 g. The latter authors also discussed the aforementioned case of *Inada & Hamazaki* as well as three others published in the Japanese literature (*Kurobane et al.* 1957, *Wada et al.* 1957, *Nakano & Shimomura* 1958), two of which previously had been diagnosed as aberrant thymoma.

DISCUSSION

There is little room for doubt that the cases I have described in this paper belong to the same category as those reported under various designations by the forementioned researchers. The most likely reason why some workers have sought genetically to associate the lesion with the thymus is its predilection for the mediastinum. All but one of the previously reported cases have been localized to the mediastinum. The exception is one of *Cohen's* two cases in which is mentioned the site of the tumour was the deltoid region. This exception and the fact that three of my cases also were extrathoracic put the thymogenic nature of the lesion in a rather doubtful light. Another reason why some workers have designated the process as a thymoma would seem to be the fact that the at least hinted concentric layering of the clusters of reticular cells centrally in the follicular foci give them an appearance which to some extent superficially resembles Hassall's corpuscles. In addition the concentrically layered perivascular hyaline seems to have been identified with Hassall's corpuscles in one of the reported cases. Accordingly I am in agreement with *Castleman et al.* (1956) in denying a genetic association between the lesion and the thymus. Conversely, it seems most reasonable to relate it to lymphoid tissue, in the first place the lymph nodes. Unfortunately, however, the lymphadenic origin cannot be proved since sinusoid patterning of the tumour in no case has been demonstrated.

So far as the nature of the process is concerned—inflammatory or neoplastic—it has already been pointed out that *Castleman et al.* asserted that it is a form of lymph node hyperplasia secondary to inflammation. For several reasons I consider this hypothesis unlikely. Firstly the germinative centres did not have the appearance usually observed in lymph nodes which are the site of a chronic inflammatory process. Secondly, it seems extremely improbable that in conjunction with the mediastinal tumours an inflammatory process in or near the mediastinum with its abundance of lymph nodes would give rise to changes confined to a single lymph node and induce such extreme enlargement of it whilst neighbouring lymph nodes are left unaffected. One of the reasons for *Castleman et al.* assumption that the lesion has an inflammatory origin was probably that in some of the cases plasma cells were

present in the lymphoid tissue Notably however, plasma cells were not demonstrable in any of my cases nor in either of the two published by *Cohen* On these grounds I am most inclined to interpret the lesion as neoplastic

Obviously, with respect to the biological character of the lesion, it should be considered benign Neither local recurrence nor metastatic spread seem to have occurred in any of the previously reported cases And in my cases signs of a recurrence are still lacking after observation for at least two and at most seven years

Considering that the lesion under discussion seems to be a benign neoplastic process, I submit that it should be named a *follicular lymphoreticuloma*

SUMMARY

The writer describes four cases of a characteristic form of lymphoid tissue proliferation Two of the patients were men and two were women The respective sites of the tumours were the axilla the supraclavicular fossa the breast and the mediastinum They were well circumscribed and amenable to surgical removal without undue difficulty and exhibited a greyish yellow, glistening cut surface Microscopically it appeared that they were composed of a follicularly patterned lympho reticular tissue with a high cell content Centrally in the follicles there were small rounded clusters of pallidly plasmatic reticular cells The interposed lympho reticular tissue was profusely vascularized and displayed delicate fibrillar hyalinization No plasma cells nor any sinusoidal patterning were observed in the tissue In a couple of cases unchanged lymph nodes were encountered in contact with the tumour The writer points out that 19 similar cases have been reported previously and alternatively interpreted as thymoma or lymph node hyperplasia However, he rejects both these interpretations and submits that the lesion more likely is a benign neoplastic process for which the term *follicular lympho reticuloma* is suggested

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Cohen H Tumor like proliferations of lymphoid tissue Occurrence in deltoid muscle and mediastinum J Mt Sinai Hosp [illegible]

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SUMMARY

The writer describes four cases of a characteristic form of lymphoid tissue proliferation Two of the patients were men and two were women The respective sites of the tumours were the axilla, the supraclavicular fossa, the breast and the mediastinum They were well circumscribed and amenable to surgical removal without undue difficulty and exhibited a greyish-yellow, glistening cut surface Microscopically it appeared that they were composed of a follicularly patterned lympho-reticular tissue with a high cell content Centrally in the follicles there were small rounded clusters of pallidly plasmatic reticular cells The interposed lympho-reticular tissue was profusely vascularized and displayed delicate fibrillar hyalinization No plasma cells nor any sinusoidal patterning were observed in the tissue In a couple of cases *unchanged lymph nodes were encountered in contact with the tumour* The writer points out that 19 similar cases have been reported previously and alternatively interpreted as thymoma or lymph node hyperplasia However, he rejects both these interpretations and submits that the lesion more likely is a benign neoplastic process for which the term *follicular lympho-reticuloma* is suggested

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FROM THE STATE SERUM INSTITUTE AND FROM THE DEPARTMENT OF PATHOLOGY, MARIA HOSPITAL UNIVERSITY OF HELSINKI FINLAND

CHROMOSOME HETEROGENEITY OF CELL LINES IN VITRO

I *Differences in the Effect of Individual Human Sera on the Chromosome Number of HeLa Cells*

By

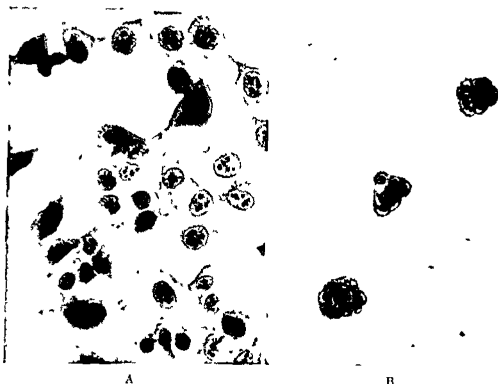
L. SAKSELA, E. SAXÉN and K. PENTTINEN

Received 19 viii 60

In a tissue culture cell strain, those cells which have the most frequently occuring chromosome number constitute what is termed the "stem line" In the cell divisions of a routine cultivated HeLa population there occur at a certain frequency abnormalities, polyploidization, multipolar spindles and other mitotic anomalies which lead to heteroploidy and aneuploidy and thus to wide variation of the chromosome number (*Hsu & Moorhead* 1956, and *Chu & Giles* 1958) The stem line chromosome number is then established from this heterogeneous genetic material through the selective forces of the environment, and it can thus be understood to be a result of a delicate equilibrium between factors [illegible]
Hiatt 19 [illegible]
1960) [illegible]
the stu[illegible] and his associates, who could by the employment of different growth media, by radiating the cells, or by means of their single cell cloning technique, establish cell lines which differed from the parental line as regards the stem line chromosome number or the frequency distribution of the chromosome numbers By using heterologous sera from different animal origin as the growth medium of HeLa cells *Vogt* (1959) also showed differences in the stem line chromosome number of the resulting cell lines

Penttinen & Saxén (1956) have shown that the behaviour of HeLa cells is different in fresh active and in heat-inactivated human serum The cells grown in these sera also showed d[illegible]
[illegible] active human sera, of which some cause

Aided by a grant from the *Sigrid Juselius Foundation*
The technical assistance of Miss *Dywcke Bremer* is gratefully acknowledged



A B

Fig 1
Differences in the growth behaviour of HeLa cells after two days growth in two different fresh active human sera In A, the loose migratory structure of cells grown in a fresh serum of the type Br is presented, and in B the "strong clumping" caused by a serum of the type Sx (Hematoxylin and eosin × 400)

what is termed "strong clumping" (Fig 1), and the others do not (*Penttinen & Saxén* 1957) The effect of the serum from the same person has been found to remain the same in repeated bleedings during a period of more than five years The sera which cause strong clumping have also some growth controlling properties, as after repeated feedings with the same fresh active serum, only a few cells are left in the tubes to which strong clumping serum has been added, whereas in the tubes in which the serum used lacks this property the cells grow well (*Penttinen & Saxen* 1959)

In this paper, results are presented which show that differences also exist in the effect of individual fresh active human sera on the distribution of chromosome numbers and on the stem line chromosome number of HeLa cells

MATERIALS AND METHODS

HeLa cells were used The cells were routine cultivated in Roux bottles contain

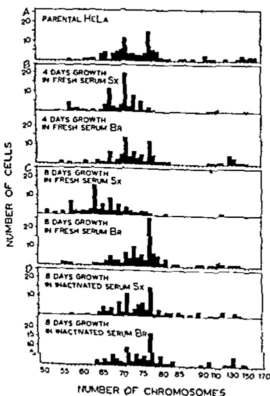
For chromosomal counts 0.1 ml Colcemid (Ciba) was added the final Colcemid

dilution being 10-6 The slides were removed after 18 hours growth Osmotic expansion and fixation of the cells were performed by the methods of *Tjio & Puck* (1958) After staining according to the Feulgen nuclear reaction the chromosomes were counted microscopically with 1200 × magnification 100 clearly distinguishable mitoses at metaphase were counted for the determination of each distribution curve of the chromosome numbers

RESULTS

Fig 2 A shows the distribution of the chromosome numbers of the routine cultivated HeLa cell line used in these experiments It has been found to remain practically the same in subsequent counts during a period exceeding one year

The results of the chromosome counts after four days growth in two different individual fresh active sera are presented in Fig 2 B No clear differences in the distribution of the chromosome numbers of the cells grown in these sera are yet to be seen However in serum Sx which causes strong clumping the cells showed a lower frequency of higher



... (A) after 4 days (B) ... n sera of the types Sx ... in the same sera in a

polyploidies and a slight shift towards lower chromosome numbers as related to the chromosome number distribution of the cells grown in serum Br, which is negative as regards its clumping ability After 8 days growth, with 8 changes of the culture medium and one trypsinization and re-seeding, the differences were marked (Fig 2 C) A clear shift towards lower chromosome numbers had occurred in serum Sx, the mean chromosome number having changed from 86 6 in the parental HeLa cell line to 64 7. The cells grown in serum Br showed no such change, the mean chromosome number being 87 5 In serum Sx, the stem line chromosome number of the cells was 64 in serum Br 78, and also the almost complete absence of the higher polyploidies indicated the action of the serum Sx Three sera of the type "Sx" and three of the type "Br" have been studied so far, and similar differences have been observed between their effects on the chromosome number distribution of HeLa cells

Parallel to these experiments, the cells were also cultivated by identical methods in the corresponding sera in heat-inactivated state (30 in 56° C. The results of the chromosome counts after 8 days' growth are presented in Fig. 2 D No differences were found in the effect of the sera Sx and Br in a heat-inactivated state

DISCUSSION AND SUMMARY

The distribution of the chromosome numbers of HeLa cells grown in fresh active human serum pool is different from that of cells grown in the same serum pool in a heat-inactivated state However, the effect of each individual active serum is not the same There are some active human sera which have a particularly strong effect in changing the distribution of the chromosome numbers, as also the stem line chromosome number of the routine cultivated HeLa cell strain and also other active sera which seem to lack this property Investigations are in progress to detect the possible connection of this phenomenon with the cancer problem

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TRANSFERRIN VARIATIONS IN SERA OF MACACA IRUS

By

LARS BECKMAN, JAN HIRSCHFELD and ULF SÖDERBERG

Received 25 viii 60

During the past years, evidence has been obtained for the occurrence of qualitative serum protein differences in human sera

By means of starch-gel electrophoresis (20, 23), *Smithies* found three serum protein patterns in human sera The different serum groups were found to be genetically determined by a pair of autosomal genes (25)

The varying proteins were later identified as the haptoglobins (26) originally described by *Polonovski & Jayle* in 1938 (15) The three serum groups were accordingly called haptoglobin 1-1 2 1 and 2 2 (26)

Further investigations of human sera by means of starch-gel electrophoresis have revealed also other genetically determined serum protein polymorphisms In 1957, *Smithies* described the occurrence of a new protein called β globulin D, which was found in two of 49 American negroes and 5 of 23 Australian aborigines (21) This component migrated slower than the normally occurring component called β globulin C, which in paper electrophoresis migrates as a β-globulin (17) The β-globulin D was not found in 425 sera from Canadian whites On the other hand 5 of these sera had a component migrating faster than the normally occuring β globulin C which was called β globulin B (22)

Further investigations showed that also other β-globulin types exist in human sera which migrate faster than or slower than β-globulin C (5 6 8 17 21 22)

Evidence for the inheritance of these different β-globulin types has also been presented (6 8 13 22) as well as for the identity of these types with human transferrin or siderophilin which is an iron binding

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From the Institute for Medical Genetics Uppsala Sweden (Head Prof Jan A ... Head Prof Axel ... ckholm (Head ... Statens Bakt ... and the Nobel ... Head Prof Ragnar Granit)

One of us (J H gratefully acknowledges grants from *Karolinska Institutet* and *Stiftelsen Therese och Johan Anderssons Minne* as well as excellent technical assistance by Miss *Berit Lindholm* Department of Bacteriology Karolinska Institutet

protein with a molecular weight of 88,000 migrating in paper and free electrophoresis as a β-globulin (1, 4, 5, 14, 18, 19, 24)

In previous investigations, serum protein differences in monkey sera (Macaca irus, F Cuv) were demonstrated by means of immunoelectrophoresis (9) and starch-gel electrophoresis (2, 3) At least four immunologically different protein systems were found to show electrophoretic variations in different sera (9), two of which were also demonstrated by means of starch-gel electrophoresis (2, 3)

One of these systems migrated as β-globulins in immunoelectrophoresis and were found to be immunologically related to human transferrin but occupying slightly faster electrophoretic positions both in immunoelectrophoresis and starch gel electrophoresis

In starch-gel electrophoresis three different β-globulins were distinguished namely sera containing a single β globulin component and sera which in addition to this component also had one of two different faster moving components (2)

In immunoelectrophoresis, these types gave precipitates which were *normally* curved, extended or two-peaked, thus suggesting an immunological identity-reaction between electrophoretically different components (2 9, 11)

MATERIALS AND METHODS

The sera from 36 *monkeys* (*Macaca irus*) were examined by starch gel electrophoresis and agar gel electrophoresis applying a previously described micro modification of agar gel electrophoresis (10) The agar gel technique allows a good resolution of components with a *high degree* of [illegible]

[illegible]

The agar gel was left to cool in a [illegible]

[illegible] (10–12)

[illegible] no screen X-ray film [illegible] agar plates [illegible]

Starc[illegible] by [illegible] strated [illegible] scintil[illegible] and using a [illegible] containing the transferrin bands were found to show a significantly increased activity as compared to slices from other parts of the gel

RESULTS

Of the 36 [illegible]

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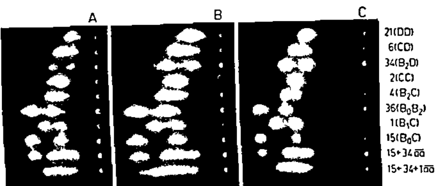


Fig 1

Trip l cate experiments of the serum types in Table 1 and mixture of sera (A C) In sli le C a slightly longer separation time was employed The electrophoretic pattern is developed by autorad ograph) Anode to the left start ng point (circles) to the r ght

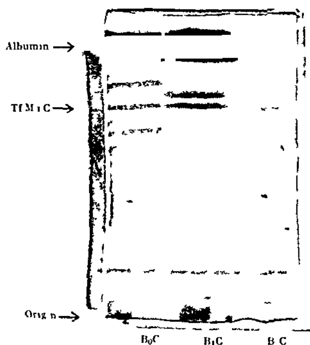


Fig 2

Photograph showing the electrophoretic patterns on starch gel of three different sera representing the transferrin types B$_0$C, B$_1$C and B C. The transferrins are located next to the all umin

fast components were observed. Three of the sera did not contain the common component. One of them had the slow component only, one had two different fast components and one had a fast and a slow component together.

It is probable that like in man the transferrin polymorphism of Macaca irus is genetically controlled. Although the main electrophoretic positions of the β-globulins in Macaca irus are different from that of humans, the polymorphic pattern seems to be of the same type. We propose the notation Tf M. i. for the transferrins of Macaca irus. M. i. stands for Macaca irus. In analogy with the notation for human transferrin variants we suggest the notation Tf M. i. C for the component occurring most frequently in the investigated sera, Tf M. i. D for the slow band and Tf M. i. B_2, B_1 and B_0 for the three different fast components, in order of increasing mobilities towards the anode.

TABLE 1

Occurrence of Different Transferrin Types Found in 36 Randomly Chosen Monkey Sera. The Individual Numbers Refer to the Order in which these Sera Were Tested and to the Sera Shown in the Figures

Transferrin type	Number	Individual numbers
CC	14	(2 5 10 13 17 18 19 20 22 23 24 31 32 33)
DD	1	(21)
B_0C	1	(15)
B_0B_2	1	(36)
B_1C	4	(1 3 8 12)
B_2C	4	(4 7 26 28)
B_2D	1	(34)
CD	10	(6 7 11 14 16 25 27 29 30 35)

In Table 1 the different combinations are listed. The appearance of the individual sera in agar-gel electrophoresis is shown by the autoradiographs in Fig. 1. Fig. 2 shows three different sera run on the same starch-gel representing the three different fast transferrin components. The transferrins (which are located next to the albumin in the picture) are thus easily demonstrated by horizontal starch-gel electrophoresis.

The monkeys rarely reproduce in captivity so that it is practically impossible to collect any representative family data to show the genetic transmission. Assuming a simple inheritance of the transferrin types in Macaca irus, however, the gene frequencies of the five different genes and the expected frequencies of the phenotypes may be calculated (Tables 2, 3). Eight out of the 15 possible combinations were observed. From Table 3 it is clear that there is a fairly good agreement between the observed and expected numbers. The numbers are in most classes too small for a significance test, however.

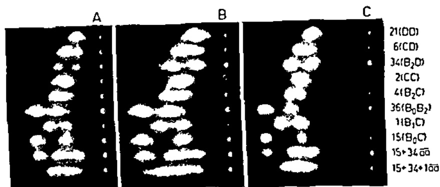


Fig 1
Triplicate experiments of the serum types in Table 1 and mixture of sera (A–C). In slide C, a slightly longer separation time was employed. The electrophoretic pattern is developed by autoradiography. Anode to the left, starting point (circles) to the right

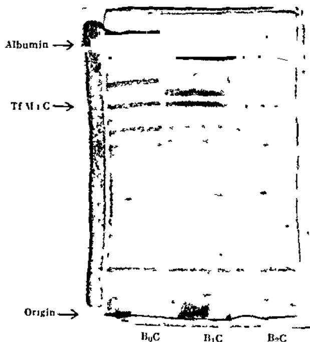


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TABLE 1

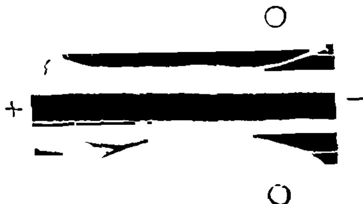
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M 1 B,C + Homo CCaa



M 1 CC + Homo CCaa

Fig 4
Immuno electrophoretic patterns of mixture of monkey sera with two or one transferrins with a normal human serum of the transferrin type CC. Three or two peaked precipitates are seen which are confluent when developed with this particular anti human immune serum prepared on rabbit

DISCUSSION

The electrophoretically different β globulins have been found to be immunological identical by means of immunoelectrophoresis In immunoelectrophoresis sera with more than one β globulin give precipitates with different shapes *e g* 2 peaked or extended precipitates whereas sera containing only one β globulin or β globulins which are very close to each other present themselves as normally are shaped or extended precipitates in immunoelectrophoresis (Fig 3) The shape of the precipitates were found to be in good agreement with the number of and distance between electrophoretically different β globulins as demonstrated by means of starch gel or agar gel electrophoresis

When these monkey sera were investigated in immunoelectrophoresis against a commercial preparation of purified anti human transferrin serum prepared on rabbits (Behring Werke) these precipitates were the only to appear and even in this instance the shape of the precipitate varied in the same way as when investigated against complete anti human immune serum (Fig 3)

Mixtures of equal amounts of human and monkey serum gave two or three peaked precipitates depending on the number of different β globulins in the monkey serum (Fig 4) These precipitates were found to depend on a partial or total confluence of the monkey β globulins with the human transferrin precipitate thus indicating a total or partial serological relationship between these components which depended on the immune serum used to develop the pattern (Fig 4 5)

TABLE 2
Expected Frequency of the Different Transferrins in Monkeys Calculated from the Investigated Material Assuming a Simple Inheritance of these Components

C	D	B_0	B_1	B_2	Total
0.653	0.181	0.028	0.055	0.083	1.000

TABLE 3
Agreement Between the Observed and Expected Frequencies of Transferrin Types Assuming a Simple Inheritance of the Different Types

Transferrin types	Observed	Expected	χ^2 1 D.F.
CC	14	15.35	0.119
B_0C	1	1.34	
B_1C	4	2.59	–
B_2C	4	3.90	
CD	10	8.51	0.261
DD	1	1.18	
B_2D	1	1.08	–
B_0B_2	1	0.17	
Seven additional combinations	0	1.88	
	36		

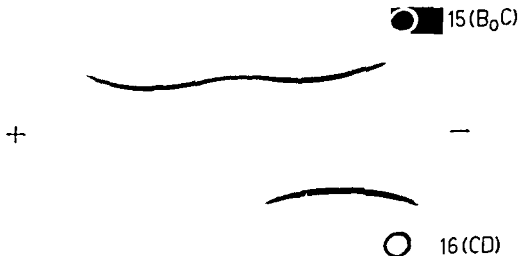


Fig. 3

Immuno electrophoretic pattern of monkey serum 15 and 16 developed with a specific anti human transferrin serum. Note two peaked shape of the precipitate from monkey serum 15 indicating a serological relation between the two components and slightly extended shape of the precipitate from monkey serum 16 which also indicates two electrophoretically different although immunologically identical components which are however too closely situated to give a two peaked precipitate.

cause of the immunological relationship demonstrated by means of mixture experiments and specific immune sera and their iron binding capacity.

It is interesting to note that in monkeys individuals with transferrin variants occur in a frequency of about 60 per cent while in Caucasians the frequency is about 1 per cent and that there is a higher frequency of transferrin variants in certain human populations as negros and Australian aborigines as compared to Caucasians (21, 22).

The fact that the mammalian serum proteins can be divided into constantly occurring qualitatively invariable fractions and variable polymorphic fractions is highly interesting both from physiological and phylogenetical points of view. As already mentioned primate sera seem to possess structurally similar polymorphic β globulin patterns in spite of their entirely different main electrophoretic positions. This indicates that β globulin polymorphism *per se* may have an adaptive value and may be a functionally important trait in transferrin metabolism.

SUMMARY

(1) The sera of 36 Cynomolgus monkeys (Macaca irus F. Cuv.) were examined by means of immunoelectrophoresis, agar gel electrophoresis and starch gel electrophoresis.

(2) Five different electrophoretically separable transferrin components were observed. 8 out of 15 possible combinations were found. The observed frequencies agreed fairly well with the expected ones assuming a single genetic transmission but the number of sera are too small to allow a statistical analysis of the data.

(3) The iron binding capacity was demonstrated by means of Fe^{59}. The five transferrins were immunologically identical in immunoelectrophoresis.

(4) The possible genetical and phylogenetical significance of β globulin polymorphism in primates is discussed.

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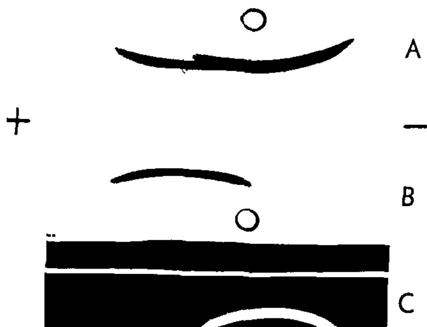


Fig. 5
Immuno electrophoretic pattern of mixture of monkey serum 16 (CD) and a normal human serum (CC) in equal amounts (A), monkey serum 16 alone (B) and human serum alone (C) developed with an anti human transferrin serum. Note differences *in electrophoretic positions of monkey and human transferrin and also that with* this immune serum a partial confluence of the precipitin arcs is obtained



Fig. 6
Immuno electrophoretic pattern of *Fe^{59} labelled monkey sera 15 and 16 developed* by autoradiographs

Addition of Fe^{59} to the monkey sera showed that these components *possessed the ability to transport in vitro* added Fe^{59} (Fig. 1, 6).

The electrophoretic mobilities of these β-globulins are slightly faster than human transferrin both in agar-gel and starch-gel electrophoresis. This is true even of the slowest component hitherto found (Fig. 5).

The variable β-globulins *in humans and monkeys may in spite of* their different electrophoretic mobilities be rather closely related be-

cause of the immunological relationship demonstrated by means of mixture experiments and specific immune sera and their iron binding capacity

It is interesting to note that in monkeys individuals with transferrin variants occur in a frequency of about 60 per cent while in Caucasians the frequency is about 1 per cent and that there is a higher frequency of transferrin variants in certain human populations as negros and Australian aborigines as compared to Caucasians (21, 22)

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STATE INSTITUTE FOR BLOOD GROUP SEROLOGY STATENS RÄTTSKEMISKA LABORATORIUM (HEAD B BROMAN M D) AND DEPARTMENT OF VIRUS RESEARCH KAROLINSKA INSTITUTET (HEAD S GARD M D) STOCKHOLM SWEDEN

INTERACTION BETWEEN HUMAN SERUM AND HUMAN FETAL CELLS GROWN IN VITRO STUDIED WITH THE AID OF THE MIXED AGGLUTINATION TECHNIQUE

By

CLAES F HÖGMAN

Received 25 viii 60

In previous communications (*Högman* 1959 a and b) it was shown that specific mixed agglutination (*Coombs et al* 1956) can be obtained between cultured human cells and human erythrocytes By use of two big serum pools, one containing anti A and the other anti-B the presence of A like and B like receptors were demonstrated on the cells of different organs and in good agreement with the blood group of the fetus The reactions occurred according to the scheme given in Table 1 As certain group O sera contain antibodies capable of reacting with both blood group A and B red cells, so called A-B cross-reacting antibodies (*Owen* 1954) a third type of reaction might be expected (cf lower part of Table 1) In the present investigation a number of human sera are tested by the mixed agglutination technique on cultured cells of human fetal kidney, lung and liver The significance of the reactions, both those being in agreement with the scheme of Table 1 and those deviating from these patterns are considered

MATERIAL AND METHODS

[illegible] tissue derived f [illegible] te cells grew in a [illegible]

[illegible] culture described in [illegible] 1959 a) The principles and the modifications of the technique were the following

(1) The cultures were washed once with phosph[illegible] 7 2 [illegible]

[illegible] latter part of this investigation 0 2 ml volumes

This study was aided by a grant from the *Swedish Cancer Society* For skilful technical assistance the author is indebted to Miss *Britt Westerdahl*

were added to each tube after the PBS had been removed by suction with a pasteur pipette. If not otherwise stated the serum was diluted with an equal volume of PBS *before incubation*.

(2) The cultures were washed three times with PBS and

(3) one ml volumes of 0.5 per cent suspensions of red cells in PBS were added to the tubes.

(4) After incubation in slightly inclined position for one hour during which the erythrocytes formed a sediment on the cultured cells *the tubes were rocked gently* to loosen the sediment and then placed in a rack.

(5) After another one or two hours the tubes were read under the low power microscope. A positive reaction consisted of adsorption of red cells around the edges and on the surface of the cultured cells. The reactions were recorded as follows: ++++ means that *more than 50 per cent of the cultured cells* were involved in mixed agglutination, +++ 5–50 per cent positive, ++ 0.5–5 per cent positive and + less than 0.5 per cent positive.

Test sera. Serum was prepared from clotted blood obtained from healthy blood donors selected at *random, donors immunized with group specific substance (Knickerbocker biosales)*, newly delivered mothers selected at random or from mothers who had newly been delivered from infants known or suspected of suffering from hemolytic disease due to anti-A or anti-B. As control sera the same pools of anti-A (No. 5) and anti-B (No. 6) were used as in previous communications.

Neutralization of sera with human saliva. Freshly obtained human saliva from group A, B and O secretors was boiled for 15 min. and then clarified by centrifugation and kept at —28° C.

Equal volumes of sensitizing serum and saliva *were mixed* and *allowed to stand* for 30 min. at room temperature. The mixture was then used in the tests.

TABLE 1

Mixed Agglutination Reaction Pattern to be Expected after Sensitization of Human Tissue Cells with Sera Containing Blood Group Antibodies

Blood group antigens on tissue cells	A			B			AB			O		
Blood group antigens of test red cells	A	B	O	A	B	O	A	B	O	A	B	O
Sensitizing serum: Group A donor Anti B	—	—	—	—	+	—	—	+	—	—	—	
Group B donor Anti A	+	—	—	—	—	—	+			—	—	
Group O donor Anti A + Anti B	+			—	+		+	+			—	—
Group AB donor No antibodies	—	—	—				—	—		—		
Group O donor with cross reacting antibody Anti AB	+	+	—	+	+	—	+	+			-	

Test red cells. Four parts of blood were mixed with one part of ACD solution (USP solution B), distributed into test tubes and kept at +4° C for a maximum period of 14 days. Before use they were washed three times in 0.9 per cent saline.

Determination of anti-A and anti-B agglutinins and hemolysins of the sensitizing sera. The hemagglutinin titres were determined by the tube method at room temperature (20–22° C) and read after two hours of sedimentation without centrifugation.

The hemolysins were determined according to *Crawford et al* (1952) As the source of complement serum from a group O donor not containing hemolysins was used It was prepared within 3 hours after bleeding and stored under dry ice for a period not exceeding 6 weeks

TABLE 2

Mixed Agglutination Obtained with Lung Tissue of Group A Fetuses After Sensitization with Group O Sera

	A or B immunized group O persons		Unselected group O mothers at delivery		Unselected group O blood donors	
	No of tests	Per cent	No of tests	Per cent	No of tests	Per cent
Reaction typical for A antigen	23	32	9	20	17	20
Atypical reaction	40	56	23	50	42	50
Negative reaction	9	12	14	30	25	30

RESULTS

The reaction pattern of mixed agglutination caused by different human sera As is mentioned in the introduction it was possible to [illegible]

reactions frequently deviated from this scheme Either did not mixed agglutination occur although the serum contained the relevant iso agglutinin or was the reaction "atypical" This meant that the sensitized cells did not only adsorb red cells of the homologous blood group but also cells of heterologous groups In the following a number of typical examples of experiments will be given illustrating different factors which influenced the reaction

The origin of the serum with regard to antigenic stimuli seemed to be of importance Table 2 shows the result of testing group O sera with lung tissue from group A fetuses A tendency towards a higher frequency of positive reactions seemed to exist in the group of A *and* B immunized persons as compared with the blood donors

The investigation of 42 group O sera from A or B immunized individuals is summarized in Table 3 In striking contrast to the previous results with the *A and B serum* pools (*Hogman* 1959 *b*) tests on kidney tissue gave reactions specific for the ABO group in 5 10 per cent only, in lung and liver cultures 30–35 per cent The majority of the positive reactions showed an "atypical" pattern But even these *reactions* seemed to be correlated to the ABO blood group of the fetuses, the frequency when tested with A and B [illegible] cent and with gro[illegible] be noted that in th[illegible] red cells (*e g* grou[illegible]ually much stronger than that of heterologous ones

TABLE 3

Investigation of 42 Sera of Group O Mothers Delivered from Group A or B Infants with Certain or Suspected Hemolytic Disease Due to Anti A or Anti B

Mixed agglutination reaction pattern Red cells			Kidney			Lung			Liver		
			Blood group of fetus			Blood group of fetus			Blood group of fetus		
A	B	O	A	B	O	A	B	O	A	B	O
+	—	—	5	–	–	23	–	–	15	–	
—	+	—	–	2	2	–	8	2	–	4	1
+	+	—	10	10	–	18	7	–	10	2	–
+	+	+	28	15	3	21	4	–	12	2	–
other types			3	2	1	1	2	–	1	–	–
—	—	—	9	9	16	9	4	27	5	1	22
No of experiments			55	38	22	72	25	29	43	9	23
Percentage of											
"specific reactions			9	5	–	32	32	–	35	–	
atypical reactions			75	71	27	56	52	7	53	–	4
negative reactions			16	24	73	12	16	93	12		96

Some sera consistently showed the same "atypical" pattern when tested on tissue from different fetuses of the same ABO group This is exemplified by the A fetuses in Table 4

TABLE 4

Mixed Agglutination Reactions after Incubation of Cultured Kidney and Lung Cells with Group O Sera

Kidney cultures Serum S I					Lung cultures Serum K				
Fetus no	Blood group of fetus	Red cells A_1	Red cells B	Red cells O	Fetus no	Blood group of fetus	Red cells A	Red cells B	Red cells O
157	A	+++	+++	+++	196	A	++++	++	
10	A	+++	+++	+++	5	A	++	+++	
72	A	++++	+++	++	205	AB	(+)	+++	
27	A	++++	++	++	194	B	++	+++	
77	A	++++	++	+	190	B	+	+++	
125	A	++++	++	+	222	B	+	+++	
39	AB	++++	++++	+++	206	B	?	+	
11	B	—	—	?	16	O	?	(+)	
40	B	+	+		2	O			
34	O	(+)	(+)	(+)	4	O			

Serum S I Group O mother with erythroblastotic group A infant
Serum K Group O donor immunized with group B substance

If thus the reaction pattern was highly dependent on the serum used for sensitization of the cells, the nature of the antigens of these cells was equally important The presence of blood group A or B like antigenic structures was indicated by the "specific reactions The cross-

reactions of the anti-AB type was also found when the tissue contained A or B antigen as was to be expected (cf Table 4, example 2).

Surprisingly, however, "atypical" reactions of the type that could not be explained by the assumption of A-B cross reacting antibodies, were often related to the ABO system In the first of the two examples in Table 4 the reaction was repeated in 7 different batches of tissue containing A antigen whereas a negative or dubious reaction occurred with group B or O tissue But a variation of the reaction pattern was also observed within one and the same ABO group as is demonstrated in Table 5

TABLE 5

Mixed Agglutination Reactions Obtained on Lung Cultures of Different Fetuses after Sensitization with a Group O Serum (F F)

Fetus no	Blood group of fetus	Red cells of group A_1	B	O
160	A	+++	+++	++
171	A	+++	+	+
213	A	+++	—	—
205	AB	+	++	+
222	B	++	+++	+
161	B	+	+++	—
206	B	—	+++	—
16	O	—	—	(+)
33	O	(+)	—	—

TABLE 6

Different Reaction Patterns Obtained with 15 Human Group O Sera on Lung and Liver Cultures of a Group AB Fetus (No 44 60)

	Lung				Liver			
	Red cells of groups				Red cells of groups			
	A_1 C H	B B F	O B M	O J J	A_1 C H	B B F	O B M	O J J
12805	+++	++++	+++	+	+	++++	—	—
S	+++	+++	+++	—	+++	+++	—	—
12803	+++	+++	+++	—	—	++	+	—
Ca	+++	—	—	—	++	+	—	—
A S	+++	+	+++	—?	+	—	—	—
G M	++	+++	++	—	—	—?	—	—
S F	+++	++	+++	—	—	—	—	—
10567	++	+	++	—	—	—	—?	—
W	++	+	+	—	—	—	—	—
N	—	+++	(+)	—	—	—	—	—
Z	—	—	—	—	—	—	—	—
10561	—	—	—	—	—	—	—	—
10565	—	—	—	—	—	—	—	—
Anti A No 5	+++	—	—	—	—	—	—	—
Anti B No 6	—	+++	—	—	—	+++	—	—

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+	—	—	5	–	–	23	–	–	15	–	–
—	+	—		2	2	–	8	2	–	4	1
+	+	—	10	10	–	18	7	–	10	2	–
+	+	+	28	15	3	21	4	–	12	2	–
other types			3	2	1	1	2	–	1	–	–
—	—	—	9	9	16	9	4	27	5	1	22
No of experiments			55	38	22	72	25	29	43	9	23
Percentage of											
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atypical reactions			75	71	27	56	52	7	53		4
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77	A	++++	++	+	190	B		+++	
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30	AB	++++	++++	+++	206	B	?	++	
11	B		—	?	16	O	?	(+)	
40	B	+	+		2	O			
34	O	(+)	(+)	(+)	4	O			

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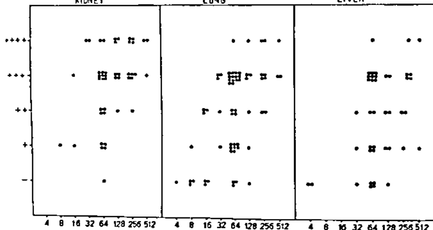


Fig 1

Comparison between mixed agglutination reaction and titre of hemagglutinin In several instances the sera were tested with tissue from different fetuses If divergent result was obtained two tests representing the highest and lowest value were used

H substance in saliva often showed negative or considerably weaker reactions than did other group O bloods It seemed to be of interest that this donor was a non secretor However, no correlation between the secretor property and the strength of mixed agglutination was demonstrated when red cells of other non secretors were tested

TABLE 8

Mixed Agglutination of Kidney Cells from a Group O Fetus and Red Cells of Group O Obtained after Sensitization with 30 Random Donor Sera

Blood group of serum donor	Kidney no 6? 60 +++	++ + (+)		Total
A	2	6	2	10
B	3	5	2	10
O	2	6	2	10
Total	23 (75 %)		6	30

It has been mentioned above that there was a correlation of the mixed agglutination and the ABO blood group of the fetal tissue even concerning the atypical reactions Most group O tissues did not show mixed agglutination This was true both if the sensitizing serum was used undiluted and as was the routine, diluted 1 in 2 However, the tissue of some group O fetuses did react, and in Table 8 cultures of this type were tested with 30 sera of blood donors of different ABO groups Positive reactions of different strength were obtained with 23 30 sera, 7 30 classified as strong No correlation to the ABO system was found in this case

In several instances the same reaction pattern was obtained when cultures from different organs of one and the same fetus were tested But this was not always the case In fact, there was a tendency of stronger reactions with kidney than with lung tissue which, in turn gave a stronger reaction than liver This may be illustrated by a parallel investigation of kidney and lung from group A fetuses kidney positive/lung positive. 30/45 sera, kidney positive/lung negative 8/45 sera, kidney negative/lung negative 7/45 sera The reaction pattern too, was sometimes different with tissue from different organs of the same fetus For example, liver might show a specific reaction whereas "atypical" reactions were obtained with kidney and lung tissue (cf Table 3) Further illustration of these differences is given in Table 6

The number of cells in each culture showing mixed agglutination varied within wide limits In the kidney cultures some sera caused mixed agglutination with close to 100 per cent of the cells whereas the frequency of positives was less in lung and liver cultures This was probably due to differences in the amount of reacting substances of the cells in a certain population As is seen in Table 6 a serum which caused a positive reaction with about 5–50 per cent of lung cells may not at all react with liver cells of the same fetus But that the liver cells were capable of reacting in the system is demonstrated by other sera causing positive reactions Note also that, in this experiment, a reaction typical for the A antigen was demonstrated on lung but not on liver tissue by means of the control serum whereas the B antigen was found in both types of tissue

TABLE 7

Mixed Agglutination Patterns Obtained with a Panel of Red Cells of Different ABO Blood Groups Tested on Kidney and Liver from a Group A Fetus

Sensitizing serum	Kidney				Liver			
	A_1	A_2	B	O	A_1	A_2	B	O
E C	4/4	2/2	2/3	6/6	4/4	2/2	3/3	6 6
S	4/4	2/2	3/3	6/6	4/4	2/2	3/3	0 6
H	4/4	2/2	3/3	6/6	4/4	2/2	3/3	0 6

Another variable was the red cell (the detector cell) Table 7 shows an experiment in which different samples of group A_1 A_2 B and O blood are added to sensitized kidney and liver cells In two of the experiments on liver no reaction occurred with 6 samples of group O red cells whereas group A and B bloods reacted The reaction may thus probably be due to A-B cross-reacting antibodies On the other hand the pattern was different with the sensitized kidney cells as all samples of red cells (even group O) reacted In other experiments there was a difference between the reacting capacity of different group O bloods One donor (J I) in our standard panel of red cells belonging to type O MN Ss P+ Lu(a+) Kk Le(a+b−) Fy(a−) cdecde non-secretor of

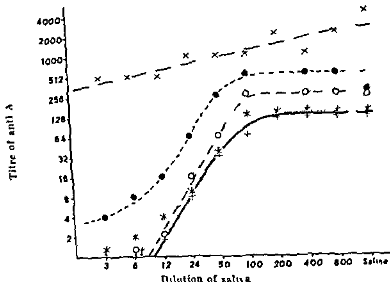


Fig 3

The inhibiting effect of varying amounts of group A saliva upon the titre of anti A measured by means of mixed agglutination and hemagglutination

+ —— + Mixed agglutination of kidney No 60 group A and red cells of group A_1
× — — × A_1 red cells } Agglutination of red cells in dextran serum medium
*   * A_2 red cells }
● - ● A_1 red cells } Agglutination of red cells in saline medium
○ — — ○ A_2 red cells }

tion reaction was obtained with saliva diluted 1 in 100 and with a dilution of 1 in 6 no reaction occurred For comparison hemagglutinin titres in saline and in dextran-serum medium (*Munk Andersen* 1956) are shown The neutralization curve for the mixed agglutination reaction has about the same shape as that of the hemagglutination of A_2 red cells in saline Similar curves of neutralization of the mixed agglutination was obtained with the control serum anti-A no 5

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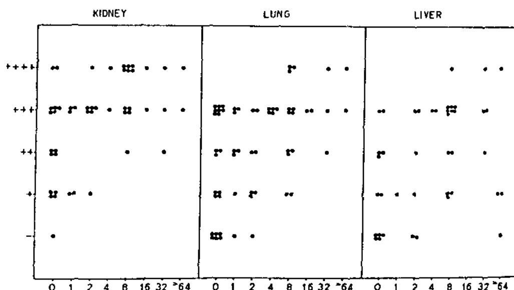


Fig 2
Comparison between mixed agglutination reaction and titre of hemolysin

The relation between mixed agglutination and the titre of anti-A and anti-B agglutinins and hemolysins The strength of the mixed agglutination reaction, mainly expressed as the relative number of sensitized cells capable of adsorbing erythrocytes, was dependent on the concentration of the reacting antibody (*Hogman* 1959 b) In order to find out if there was a correlation to the isoantibodies of the sera used the titres of anti-A and anti-B agglutinins were determined and the result is compared with the mixed agglutination reactions in Fig 1 It is seen that a serum with a titre of 64 might give a strongly positive as well as a negative mixed agglutination No close correlation seems to exist between the mixed agglutination reaction and the titre of hemagglutinin of the sensitizing sera

As the frequency of positive reactions was higher with sera from immunized persons than with donors taken at random a comparison was also made with the anti-A and anti-B hemolysins (see Fig 2) However strong positive reactions frequently occurred even with sera without detectable amounts of hemolysin On the other hand with few exceptions the reaction was positive if hemolysins were present in the serum

This lack of correspondence between the mixed agglutination and the blood group iso-agglutinins and hemolysins may not be too surprizing in the light of the differing reaction patterns demonstrated previously in this paper indicating a great inhomogeneity of the reacting substance

Inhibition experiments with human saliva If group A secretor saliva was mixed with serum containing anti-A before sensitization the mixed agglutination was expected to be inhibited Fig 3 shows the effect of varying amounts of group A saliva mixed with a group O serum containing "immune" anti-A A beginning effect upon the mixed agglutina-

Fig 3

The inhibiting effect of varying amounts of group A saliva upon the titre of anti A measured by means of mixed agglutination and hemagglutination

+ —— +	Mixed agglutination of kidney No 60 group A, and red cells of group A_1	
× — — ×	A_1 red cells	Agglutination of red cells in dextran serum medium
* *	A_2 red cells	
● - ●	A_1 red cells	Agglutination of red cells in saline medium
○ — — ○	A_2 red cells	

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TABLE 9

Inhibition of Mixed Agglutination by means of Human Group A and B Secretor Saliva

Kidney	Sensitizing serum	Serum + saline			Serum + group A saliva (CH) *			Serum + group B saliva (T R) *		
		Red cells								
		A_1 C H	B B I	O B M	A_1 C H	B B F	O B M	A_1 C H	B B I	O B M
No 187 Group A	E F	++++	+++	+++	+++	+++	+++	+++	+++	+++
	E M	++++	++++	++++	—	—	—	++++	++++	++++
	Alt	+++	+++	+++	+	+	—	++	+	+
	Anti A No 5	++++		—	—	—	—	++++	—	—
	Anti B No 6	—		—						
No 206 Group B	F I	(+)	(+)	(+)	—	(+)	—	—	—	—
	E M	—	(+)	—	—	—	—	—	—	—
	Alt	+++	+++	+++	++	+++	+++	—	—	—
	Anti A No 5									
	Anti B No 6		+++	—	—	++	—	—	—	—
No 189 Group AB	E I	+++	+++	+++	++	+++	+++	+++	+++	+++
	E M	+++	+++	+++	—	—	+++	++++	+++	++++
	Alt	++++	++++	++++	++++	++++	+	(+)	(+)	+++
	Anti A No 5	++++		(+)	—	—	(+)	++++	—	(+)
	Anti B No 6		+++		—	++		—	—	

* Dilution of saliva Experiment 187 and 206 1 in 2
Experiment 189 1 in 10

TABLE 10

Inhibition of Mixed Agglutination by means of Human Group A, B and O Secretor Saliva. The Effect upon a Group O Serum with Strong Anti B Tested on Group B Tissue

			A CH	A_2 AT	B TR	O BM	O A	O FW	O JJ
Group B Fetus No 7/60	Kidney	Saline	++++	++++	++++	++++	++++	+++	+++
		A saliva (CH)	++	++	++++	+	++	—	–
		B saliva (TR)	—	—	—	—	—	—	—?
		O saliva (KN)	++++	++	++++	—	—	—	—
	Lung	Saline	+++	+++	++++	+++	+++	+++	++
		A saliva (CH)		++	++++	++	++	+	—
		B saliva (TR)	—	—	—	++	—	—	—
		O saliva (KN)	++	++	++++	—	—	—	—
	Liver	Saline	+++	+++	++++	++	—	—	—?
		A saliva (CH)	(+)	—	+++	—?	(+)	—	—
		B saliva (TR)	—		—	—?	—	—	—
		O saliva (KN)	+++	++	+++	—	—	—	—

Three volumes of saliva and one volume of PBS were added to one volume of Serum (KB). In the saline control PBS replaced the saliva

In the experiment with group AB tissue shown in Table 9 a smaller amount of saliva was used *for neutralization* In this case the homologous reactions seemed to be inhibited to a higher degree than the cross-reaction with group O red cells

Another example is demonstrated in Table 10, concerning a group O serum from a mother (K B) of a stillborn infant of group B with signs of severe erythroblastosis The mother had a strong "immune" anti B but no other irregular antibodies The serum was tested with kidney lung, and liver tissue from a group B fetus Group B secretor saliva *inhibited all activity except that on lung tissue* where a weak reaction with one sample of group O red cells remained Group O saliva inhibited some of the cross-reactions, *i e* those involving group O red cells, but not the reactions with group A_1, A_2 and B cells Group A saliva weakened the reaction with group A and O red cells but left the reaction with B red cells apparently intact

From these experiments it may be concluded that human saliva may completely inhibit the mixed agglutination Depending on the sensitizing serum and the blood group of the tissue on which the test is carried out, group A or B saliva may be active If cross-reactions are present these are most often inhibited together with the homologous reaction In some cases heterologous saliva does not influence the reaction, in other cases it is possible to inhibit the cross-reaction (involving group O red cells) selectively by the use of group O human saliva

DISCUSSION

The reaction patterns obtained in the mixed agglutination tests performed with human fetal cells and human erythrocytes may be divided into four types

1 *Negative reactions* These may be encountered even when a positive reaction is expected and show that the human iso-agglutinins do not always cause mixed agglutination They are more frequently seen with serum from blood donors selected at random than with that of an A or B *immunized person* No close correlation to the titre of A and B hemagglutinins or hemolysins seems to exist however In this connexion the findings of *Gurner & Coombs* (1958) may be noted They state that on human leucocytes the A or B antigens are only demonstrable by the use of sera containing "immune" A and B antibodies

2 *Specific positive reactions* Apparently then mixed agglutination specific for blood group A and B antigens is obtainable only with selected sera However, according to the present writer's experience sufficiently large pools of high titering blood group A and B sera respectively have been found to yield satisfactory results

3 *Cross-reactions* Several group O sera cause mixed agglutination in which cells from a group A fetus agglutinate not only together with group A red cells but also with group B cells Group O red cells are

not involved Since the first observation by *Moss* (1910) that group A red cells might remove not only the anti A but also some of the anti B activity out of a group O serum this subject has been extensively studied by several authors (*Landsteiner & Witt* 1926 *Dodd* 1952 *Owen* 1954 *Jones & Kaneb* 1960) It has been postulated that certain group O sera contain an antibody anti C capable of reacting with an antigen C present on red cells of group A B and AB individuals but lacking in that of group O persons The second theory is that some of the antibody molecules of group O sera have an anti AB specificity and would react with the ordinary A or B receptor of the red cell Recent investigations by *Jones & Kaneb* (1960) speek in favour of the last theory They showed that the ability of red cells to accept the cross reacting antibody was impaired if the cells had been previously blocked by antibody of non cross reacting type But to explain the reaction pattern A+ B+ O— obtained in the present investigation as being true A B cross reactions it is not possible to assume that the peculiarity is only affecting the antibody One and the same serum tested on different tissues of the same fetus but under otherwise identical conditions (the same detector red cells tests performed simultaneously) may show a cross reaction on kidney cells but a specific reaction on lung or liver cells This must signify that the antigenic structure is also of importance

4 *Reactions due to serological adhesion* The most confusing reactions are those in which not only group A or B but also group O red cells are involved in mixed agglutination The phenomenon was most frequently seen on kidney tissue less often on cells derived from lung or liver The explanation first coming in mind that the cultured cells adsorbed red cells unspecifically could be excluded for several reasons

(a) The atypical reaction was only observed after sensitization with certain sera So it was not due to a panagglutinability of the non sensitized cultured cells or of the red cells

(b) A positive serum caused the same type of reaction with several different batches of tissue cultures but not all

(c) The atypical reaction was obtained in 90 125 (72 per cent) of tests with group A and B tissue but only in 9 74 (12 per cent) of tests with group O tissue (cf Table 3) Thus the reaction seemed to be associated with the presence of the A B antigens of the tissues

(d) The reaction was selectively inhibited by human saliva containing group specific substance corresponding to the type of immunization to which the serum donor had been exposed (cf Tables 9 and 10)

The phenomenon may fall into the group of reactions desc b a serological or ~
review on this

of the phenomenon is hitherto unexplained If the hypothesis is correct that some of the "atypical" reactions are due to serological adhesion the question remains what is the specificity of the antibody? The significant dominance of positive reactions obtained with tissues from group A, B and AB fetuses may indicate that A and B antibodies are involved On the other hand reactions obtained with group O tissue cannot be explained in this way Several sera *causing that type of reaction* were investigated for the presence of other blood group antibodies by means of usual hemato-serological methods with negative results If the reactions are of a specific nature, apparently, antigens not classifiable as conventional blood group antigens must be assumed to have been involved

Another factor which should be discussed is the influence of cultivation as a possible cause of the "atypical" reactions Primary explants of trypsinized tissue were used, the age *in vitro* usually not exceeding two weeks In a previous paper (*Hogman* 1960) it was shown that the capacity of mixed agglutination was lost if the cells were grown in *serial passages But this loss did not appear until after 5–9 passages* (33–71 days) In every experiment in the present investigation it was controlled by standard anti-A and anti-B sera that the tissue was capable of giving specific mixed agglutination The variation in age of the cultures did not seem to be of any importance for the occurrence of "atypical" reactions, nor did the age of the fetuses have any influence The trypsinization procedure may also play a role in changing the reactivity of the cells As is well known in blood group serology some of the antigenic structures may be destroyed However, the A and B receptors of the red cells are comparatively resistant to trypsin treatment Furthermore, in another study (Hogman in preparation) evi*dence was obtained that surface antigen is continuously produced* by the cells during cultivation A defective antigenicity caused by trypsinization is therefore probably only transcient

SUMMARY

By use of mixed agglutination applied to human fetal kidney lung and liver cells grown in tissue culture the reaction pattern of different human sera was studied

(1) All sera containing blood group A and B antibodies used for sensitization of the cells did not cause positive reactions the sera *from A and B* immunized individuals showing a higher frequency than those from unselected blood donors

(2) With one big pool of anti-A and one of anti-B serum specific reactions were obtained in agreement with the blood group of the corresponding fetus *In contrast* were the findings with individual sera showing only 5–10 per cent of group specific reactions with kidney and 30–35 per cent with lung and liver tissue

(3) By the use of immune sera positive reactions occurred in 75 90 per cent but a majority of the reactions showed an atypical reaction pattern This consisted of adsorption of both homologous and heterologous red cells on the sensitized tissue Some of these reactions could be explained as due to cross reacting antibodies of anti AB specificity, other could not be explained in this way

(4) The atypical reactions occurred in a significantly higher frequency with tissue from group A and B fetuses than from group O fetuses

(5) The reaction was often strongly assymetric in behaviour, meaning that a positive reaction with the "atypical' agglutination pattern occurred with *e g* group A tissue but not with group B or O tissue In several instances the assymetric reaction could be related to the type of immunization that the donor of the sensitizing serum had been exposed to

(6) No close correlation was found between the positive reactions and the titre of anti A and anti B hemagglutinin and hemolysin of the sensitizing sera

(7) It was possible to inhibit selectively the mixed agglutination reaction both that with the specific and that with the atypical pattern by means of human saliva of different ABO groups

It is possible that some of the reactions observed belong in the group of serological or immune adhesion The possibility of participation of non blood group antigens is also discussed

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THE DEPARTMENTS OF PHARMACOLOGY AND BACTERIOLOGY ROYAL VETERINARY COLLEGE STOCKHOLM 51 SWEDEN

AN AUTORADIOGRAPHIC STUDY OF RADIOACTIVELY LABELLED *BACILLUS CEREUS* IN THE MOUSE

By

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CARL G SCHMITERLÖW

Received 25 VIII 60

In view of the complex relationships between the host and parasite new methods for studying the distribution and fate of the organisms involved are essential to elucidate the events taking place in a given infectious disease process With this end in view, an attempt was made to exploit the whole body autoradiographic technique involving the use of radioactively labelled bacteria which as far as is known has not yet been adopted for this purpose This method affords the possibility of comparing the relative accumulation of bacteria in all the organs and tissues of the body

In the past studies of this nature required long and laborious procedures in which sampling was frequent and in which viable counts of microorganisms in each organ was necessary in order to get a relative picture of the distribution The present study was carried out in order to ascertain if the same kinds of information could be obtained in an easier manner If so then it could possibly be adopted for general use in the study of experimentally induced infections

MATERIALS AND METHODS

Mice infected with S^{35} labelled *Bacillus cereus* were chosen as the host parasite model This system was employed for two reasons 1) the organism was found to incorporate S^{35} from an inorganic source so that bacterial preparations with a high specific activity could be easily obtained and 2) the organism which is easy to cultivate is non pathogenic for humans making them less dangerous to handle and the strains employed in this investigation were uniformly pathogenic for mice

Labelling of Organisms

The strains of *B cereus* used were *B cereus* 1818 and *B cereus* 8234 according to the designation of the department of Bacteriology Royal Veterinary College Stockholm The composition of the *medium* employed for growth of the bacilli was as

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Fig 1
Autoradiographic distribution of S^{35} labelled *Bacillus cereus* in the mouse immediately after intravenous injection White areas correspond to radioactive foci

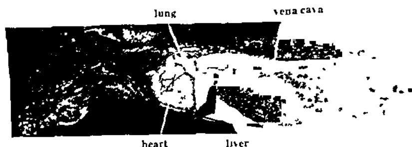


Fig 2
Autoradiographic distribution of S^{35} labelled *Bacillus cereus* in the mouse approximately 1 minute after intravenous injection

Bacillus cereus immediately after injection This in effect means that the animal was sacrified as soon as possible after completion of the intravenous inoculation From the picture it is evident that the organisms are present only in the heart and blood circulation and are beginning to accumulate in the liver If the interval between the time of injection and sacrificing is slightly prolonged, the picture obtained is already changed Fig 2 (approximately 1 minute after injection) shows that the bacteria have already begun to leave the blood stream and there is a pronounced accumulation of radioactivity in the liver, spleen and lung Furthermore, the activity is much more generalized as can be seen by the stippled appearance throughout the section This activity is probably due to the presence of organisms in the capillaries These findings are in accord with those obtained by classical microbiological methods According to *Rogers* (1959) when bacteria begin to leave the blood stream they accumulate primarily in the liver and spleen These two organs are able to sequester between 60–95 per cent of the organisms leaving the blood stream The radioactivity demonstrable in the lung so soon after inoculation would suggest that this organ is also quite active in accumulating organisms

The distribution after 15 minutes is shown in Fig 3 After this period

follows (all figures are expressed in terms of grams/liter): $MgSO_4$ 0.01, K_2HPO_4 0.87, KH_2PO_4–0.68, NaCl–1.0, NH_4Cl–0.5, NH_4HPO_4–0.5, $CaCl_2$–0.02, peptone (Difco) 0.1, glucose 2.0, $FeSO_4$ 0.02. The medium was adjusted to pH 7.6 and autoclaved for 30 minutes at 121° C. Glucose and $FeSO_4$ were sterilized and added separately in order to avoid precipitation of the salts. To the medium, which was dispensed in 48 ml quantities in 300 ml Erlenmeyer flasks, carrier free S^{35} was added so that the concentration of radioactivity was approximately 5 microcuries/ml. It was found in preliminary experiments that large quantities of organic compounds in the medium inhibited the incorporation of the inorganic S^{35}. Consequently, the semi-synthetic medium described above was employed. The small quantity of peptone added stimulated growth but did not seem to diminish the incorporation of the radioactive isotope.

The flasks were inoculated with 1.0 ml of a 24 hour culture of *B. cereus* grown in the same medium as mentioned above but without the addition of S^{35}. The cultures were then incubated at 37° C in a shaker (120 strokes/minute) for 24 hours. The cells were then harvested by centrifugation, resuspended and washed three times in physiological saline. The final cell suspension used for injection into mice was concentrated approximately five times ($\cong 10^9$/ml).

The radioactivity of the cells was measured in a Geiger-Müller counter. It was found that the activity of the final wash fluid was negligible and that the activity of the cell suspension was due to the incorporation of S^{35} into the bacterial protoplasm. The radioactivity of suspensions prepared in this manner was found to be approximately 50 000 cpm/0.1 ml. The presence of S^{35} in the growth medium had no apparent effect on the growth and viability of the microorganisms.

Injection of S^{35} Labelled Organisms in Mice

White mice weighing $\cong$ 20 g were used throughout the investigation. 0.3 ml of the labelled cell suspension was injected into the tail vein and the animals were sacrificed at various time intervals after inoculation. The mice were killed by immersion in a mixture of acetone and dry ice having a temperature of − 78° C.

Autoradiographic Technique

The mice killed instantaneously as described above were then kept in a cold room at − 10° C until ready for sectioning. The technique of whole animal sectioning, the apposition of the sections on film (Ilford G-5) and the subsequent development of the autoradiograms were carried out according to the methods described in detail by *Ullberg* (1954). In the present study the thickness of the sections was 20 microns and the time of exposure of film to the section was approximately 30 days. The positive identification of individual organs and tissues was secured by comparing the autoradiograms with the original sections stained by conventional histological techniques. In the original autoradiograms the areas of blackening correspond to tissues and organs containing radioactivity. These films were used as negatives for the reproduction of the distribution pictures. In the latter the light areas therefore correspond to the radioactive foci.

EXPERIMENTAL RESULTS

The results presented here are representative of a series of experiments. In order to present the material in a logical sequence the autoradiograms shown are those obtained using animals sacrificed at intervals from immediately after up to six hours after intravenous injection of the labelled bacilli. Six hours was found to be the maximum time of survival for any given experiment. The death of the mice was due to the infection as shown by the fact that control mice inoculated with S^{35} alone were unaffected and that pure cultures of *B. cereus* could be obtained from the blood and organs of infected animals.

Fig. 1 is an autoradiogram showing the distribution of S^{35} labelled

absence of activity in the central nervous system indicating the efficiency of the blood brain barrier

Six hours after injection (before death of the animal) there was much less activity in the lung and a great deal more in the gut As before however the picture is dominated by the activity of the liver and spleen indicating that these two organs may reseed the circulation during the final bacteremia The gradual accumulation of radioactivity seen in the bone marrow presents the interesting possibility that the reticulo endothelial cells there are active in the trapping and destruction of organisms during the initial bacteremia This observation may be significant and should be investigated thoroughly

DISCUSSION

The use of radioactive isotopes to determine the distribution and fate of many materials has done much to advance knowledge in diverse areas of biology and medicine Employed with discretion and caution there is no doubt that it is an invaluable tool However interpretation of results at times is very difficult because of the inherent nature of the method The main problem lies in distinguishing between the materials under investigation and their metabolic products In the study of organic molecules of known structure there is little danger of misinterpretation since the decomposition products can be distinguished from the original compound by means of chromatographic methods Protein molecules which are subject to denaturation and breakdown present more difficult problems and in the present study where viable replicating entities are involved the difficulties are increased still further In addition to the destruction of the organisms by the cellular and humoral factors present in the host which of course may result in the liberation of labelled catabolic products one must also keep in mind the dilution effect of activity in each bacterium during the multiplication process *in vivo*

The investigation described here would seem to indicate that the results obtained using the autoradiographic technique do not conflict with those obtained using purely microbiological methods The very rapid disappearance of bacteria from the blood stream and their accumulation in the liver spleen and lung is demonstrated in the autoradiographic pictures While the results are only qualitative it is quite possible to make them at least semi quantitative by measuring the intensity of the blackening on the original autoradiographs This can be accomplished by using a constant development time for the autoradiographs and a series of standards of known radioactivity These then could be compared in a densitometer

The accumulation of activity in the kidney and bladder very shortly after injection with *B cereus* is quite surprising since the kidney is not usually considered very active in the clearance of bacteria from the

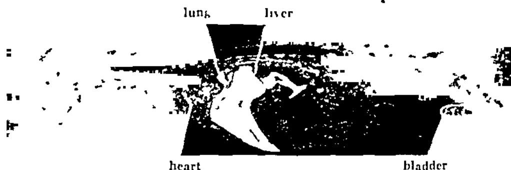


Fig 3
Autoradiographic distribution of S^{35} labelled *Bacillus cereus* in the mouse 15 minutes after intravenous injection

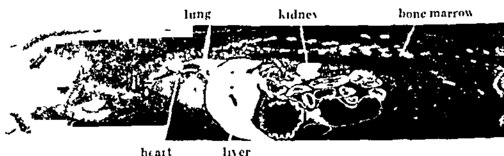


Fig 4
Autoradiographic distribution of S^{35} labelled *Bacillus cereus* in the mouse 3 hours after intravenous injection

of time, the bacilli are much less predominant in the heart whereas the liver, lung and spleen now show an intense amount of activity The activity of the lung seems to be much more apparent here than obtained by *Thorbecke* and *Benacerraf* (1959) who used P^{32}- labelled staphylococci and *Escherichia coli* in the mouse It may be that the difference is due to a species variation and that Gram positive bacilli are taken up by the lung more avidly than the cocci and Gram negative rods *Rogers* (1959) states that the lung at times may be an active site of bacterial filtration due to the leucocyte deposition within that organ

In addition there appears to be a significant amount of radioactivity present in the bladder after 15 minutes This is also true for the cortex of the kidney although this organ does not appear in this particular section It is also interesting to note that while the lymph nodes are enlarged there is no uptake of radioactivity

It was found that 1 hour after injection there was almost a disappearance of activity from the heart and blood circulation and the autoradiograms were dominated by activity in the lung liver and spleen There were also signs of accumulation in the walls of the gut Between 1 and 3 hours, the autoradiograms showed a gradual accumulation of activity in the intestinal walls and a decline of radioactivity in the lungs Fig 4 illustrates the distribution 3 hours after infection In the kidney the activity was confined primarily to the cortex There was a complete

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blood stream. The short time between injection and detection of such activity would speak against the possibility that this is due to microbial decomposition products. *Hansson* (1959) found that S^{35} labelled methionine injected *intravenously in the mouse accumulated very markedly* in the pancreas. This was not the case in the mice injected with S^{35} labelled *B. cereus* and thus would indicate that no appreciable quantities of free methionine were present. In addition the autoradiograms obtained when free S^{35} is injected is in no way similar to those obtained in this study. The distribution is quite different and the amount of radioactivity found in the kidney and bladder is much less than found when labelled bacilli were injected. One explanation for the high amount of radioactivity found in the kidney cortex is that the massive number of organisms used in experimental infections results in intravascular clumping and that the clumps then lodge in the glomeruli of the kidney. A partial destruction of the epithelium would then allow some of the bacilli to invade the tubules and ultimately appear in the bladder.

The final bacteremia before death of the mice is not apparent in the autoradiographs. This may be due to the fact that the radioactivity of the bacilli was reduced during multiplication within the tissues so that it could no longer be detected on the autoradiograms. However it may be possible to label the bacteria so that the specific activity is high enough to counteract this dilution effect.

It should be pointed out that there is a gradual accumulation of activity which most likely does not represent viable organisms. This is apparent after a few hours by the low grade activity found throughout the section and especially in the brown fat, salivary gland and skin. The activity in these tissues is probably due to the liberation of soluble products resulting from the destruction of the organisms by the defence mechanisms of the host.

In full awareness of the difficulties involved in interpretation of results, it seems likely that the autoradiographic technique described *here can be used to advantage in the study of experimental infections* and more broadly in the general problem of host parasite interaction.

SUMMARY

An autoradiographic technique for the study of infectious processes is described. Strains of *B. cereus* pathogenic for mice were labelled with S^{35} and injected intravenously. A study of the distribution of the *organisms from the blood stream to body organs showed that the in*formation obtained does not conflict with results of investigations using purely microbiological techniques. It is hoped that the autoradiographic method can be adopted for the study of experimental infections

MATERIALS AND METHODS

Strain. A highly virulent strain of *Salmonella typhimurium* designated 395 M was used[1]. It was isolated during an epidemic in 1952 and has been used for standard vaccine production.

Media. The composition of the media used is given below. The H medium is described by *Hook et al.* (1946) and the F medium by *Friedlein* (1928). The figures given represent gram per litre of medium.

Nutrient broth	Beef extract (Difco)	3
	Peptone (Difco)	5
CaY medium	Casamino acids (Difco)	30
	Yeast extract (Difco)	3
CaY G medium	Casamino acids (Difco)	10
	Yeast extract (Difco)	3
	NH_4Cl	0.5
	$Na_2HPO_4 \cdot 2H_2O$	7.5
	KH_2PO_4	3.0
	$MgSO_4 \cdot 7H_2O$	0.2
	Glucose	2.0
H medium	Glucose	4.0
	NH_4Cl	1.0
	$Na_2HPO_4 \cdot 2H_2O$	7.5
	KH_2PO_4	3.0
	NaCl	0.5
	$MgSO_4 \cdot 7H_2O$	0.2
F medium	Sodium lactate	10
	NH_4Cl	1.0
	K_2HPO_4	0.7
	KH_2PO_4	0.3
	$Na_2SO_4 \cdot 10H_2O$	0.25
	$MgSO_4 \cdot 7H_2O$	0.02

Synthetic medium to obtain high yields (nitrogen content allowing a yield of about 10 g dry weight per litre of culture).

Glucose	10
Lactic acid	20
NH_4OH	4
K_2HPO_4	7
KH_2PO_4	3
$Na_2SO_4 \cdot 10H_2O$	1.25
$MgSO_4 \cdot 7H_2O$	0.1

Glucose and magnesium sulphate were sterilized separately. After sterilization the pH was adjusted to 7.2 by adding 0.1 M sodium hydroxide (H and F media) or NH_4OH (high yield medium). An amount of a standard trace elements solution was also added to the synthetic media before inoculation.

Cultivation Techniques



[1] We are indebted to dr *H. Bilou lelle* the State Bacteriological Laboratory, Stockholm, for the supply of this strain.

FROM THE DEPARTMENT OF BACTERIOLOGY, KAROLINSKA INSTITUTET STOCKHOLM (HEAD PROF BERNDT MALMGREN M D)

PREPARATION OF BIOLOGICALLY ACTIVE FRACTIONS OF SALMONELLA TYPHIMURIUM

1 Cultivation Methods

By

T HOLME and L EDEBO

Received 5 ix 60

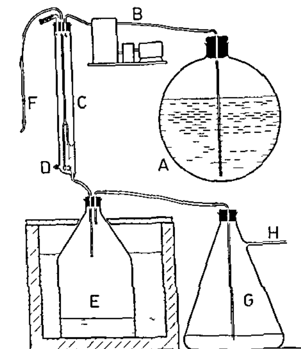
A rational approach to the problems involved in the fractionation of pathogenic microorganisms, and the study of the immunological properties of the fractions obtained, implies the elaboration of suitable cultivation methods for the preparation of cells with fully retained immunogenicity, virulence and toxicity It is a well-known fact, that the chemical composition of microorganisms is profoundly influenced by the environmental conditions of the cells Investigations concerned with changes in immunological properties of bacterial cells as a result of different cultivation conditions are relatively few in numbers and no conclusive results have been obtained (*Czerniawski et al* 1958 *Formal et al 1956, Freeman et al* 1940) A simple synthetic medium is highly preferable in basic work of this kind, since it offers an excellent basis for further studies on the influence of environmental factors on the immunological properties of the cells

When fractionation work is involved as for example the isolation and study of different cell components, fairly large amounts of material may be required A high yield of bacteria in the cultures is therefore desirable since it reduces the demand for large culture volumes which in turn, facilitates the apparatus design and the harvesting procedures

In the present paper studies on the submerged cultivation of Salmonella typhimurium, in batch culture as well as in continuous culture, are reported The work was undertaken with the aim of preparing material for fractionation of the bacterial cells and of studying the biological activity of the fractions A simple synthetic medium could be shown to give high yields of bacteria if sufficient aeration was provided No significant change in toxicity or virulence of the cells was observed when different media and cultivation techniques were tested

Our thanks are due to Miss *Kerstin Lindstedt* and Miss *Ulla Spet* for their skilled technical assistance

This work was aided by a grant from the *Swedish State Medical Research Council*



0 100 200 300 400 500 mm

Fig 1 a

Equipment used for the continuous cultivation of Salmonella typhimurium. A 40 litre storage flask for fresh medium. B Hose pressure pump — C Culture vessel. D Rubber membrane for drawing small samples. E Cooled 20 litre pyrex flask — F Air flow meter. G Suction flask with concentrated sulphuric acid. H Connection to water jet.

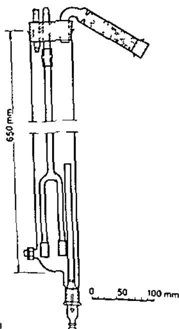


Fig 1 b

Enlargement of the culture vessel (C in Fig 1 a)

perforated by glass tubes for inlet and outlet air The ingoing air was conducted through a gas flow meter and was drawn by suction from a water jet, connected to the culture vessel via a suction flask containing concentrated sulphuric acid

TABLE 1

Aeration Efficiency as Estimated by the Sulphite Oxidation Method Obtained in the Different Culture Vessels Used for Cultivation Studies on Salmonella typhimurium

	Vessel	Volume of medium (l)	Aeration procedure	Air flow l/min	Effective aeration mM O/l h
1	6 l Erlenmeyer	3	shaken	–	<1
2	6 l	3	shaken sintered glass disc, P	3	20
3	6 l ,	3	shaken 2 sintered glass cyl P	3	110
4	6 l indented Erlenmeyer	3	shaken 2 sintered glass cyl P	3	270
5	Glass cylinder diam 70 mm	0 4	perforated glass tube P	2	10
6		0 4	1 sintered glass cylinder P	2	260
7		0 4	2 sintered glass cylinders P	2	960
8	,	0 4	3 sintered glass cylinders P	2	1000
9		0 4	3 sintered glass cylinders P	0 5	120
10		0 4	3 sintered glass cylinders P	1	360
11	,	0 4	3 sintered glass cylinders S	2	1100
12	,	0 4	3 sintered glass cylinders IP pulsat aerat 23 pulses per min	2	160

Vessels 1–4 were used for batch cultivation, the cylindrical glass apparatus was used for continuous cultivation

P = aeration under positive pressure
S = aeration under negative pressure (by suction)
IP = intermittent aeration under positive pressure (pulsating aeration)

For inoculation, precultures of 100 200 ml were cultivated in 2 3 litre Erlenmeyer flasks on a rotary shaker for 18 hours The precultures were grown in the same medium as the culture to be inoculated The amount of inoculated cells was standardized by nephelometry to correspond to a density of 10^8 cells per ml

Continuous cultures The continuous culture apparatus used is presented in Fig 1 As in the batch cultures the cultivation and sampling vessels were operated under negative pressure for safety reasons (*Gerhardt* 1946) The apparatus was placed in a constant temperature room at 37° C Good mixing was achieved in the culture by the stream of air bubbles emanating from the gas distribution cylinders However the vessel was required to have a relatively small diameter The dimensions of the culture vessel are given in Fig 1 b The culture volume can be varied between 200 and 1000 ml by exchanging the levelling tube

The same time schedule as that previously described for E coli may be used for starting a continuous run (*Holme* 1957)

Determination of the total number of cells per unit volume was made by counting in a counting chamber under the phase contrast microscope Viable count was performed by spreading 0 1 ml of tenfold dilutions of the samples on the surface of each two nutrient agar plates Dry weight determinations were made on cells washed twice in 0 01 M phosphate buffer

Determinations of aeration efficiency By using the sulphite oxidation method of *Cooper Fernstrom & Miller* (1944) it was possible to obtain a comparison between the aeration efficiency obtained with the different equipments employed In this method the rate of oxidation of sodium sulphite is measured by a titrimetric method in samples taken from the culture vessel at different times after the beginning of the aeration The results of these determinations are given in Table 1

Mouse toxicity and virulence tests Cells were killed by exposure to 0 4 per cent formaldehyde Toxicity was determined by intraperitoneal injection of 0 1 ml of twofold dilutions of the preparations, each dilution was injected into five mice

stationary phase of growth The results are presented in Table 3. LD_{50} varies between 10 and 92 but all values are within 35 per cent confidence limits

TABLE 3

Virulence for Mice of Salmonella typhimurium Cells Grown in Different Media and from Different Phases of Growth

Medium	Time after inoculation in hours	Total number of cells pr ml $\times 10^{-9}$	Number of viable cells pr ml $\times 10^{-9}$	LD_{50} (viable cells)
Nutrient broth	5	3	1.2	92
	16.5	5	1.5	24
(a) medium	5	5	2.4	59
	10	9	2.7	60
H medium	5	4	1.8	37
	16.5	8	2.8	18
F medium	5	5	1.5	10
	16.5	16	5.6	11

The yield of bacteria *i e* the dry weight of cells per unit volume in the stationary phase was increased in most cases by aerating the culture The two complex media based on casamino acids used in this experiment both gave higher yield than the synthetic media (Table 2). However in the F-medium as originally described, growth is limited at the indicated yield by exhaustion of the nitrogen source and not by oxygen deficiency Therefore, a simple synthetic medium, based on the F medium was developed where the concentrations of the different essential nutrients were increased so as to give a maximal yield of about 10 g dry weight of cells per litre of culture Several experiments, using the culture vessel 4 of Table 1, showed that the growth of S typhimurium was seriously affected at pH levels below 6 and above 8 In order to obtain maximum yield, the procedure shown in Table 4 was adopted where glucose was added during the last 5 hours of the cultivation period in order to keep the pH below 7.5

TABLE 4

Two Litre Culture of Salmonella typhimurium Grown under Active Aeration in an Indented 6 Litre Erlenmeyer Flask (vessel 5 Table 1)

Time after inoculation in hours	pH	Amount of glucose added (g l)
0	7.2	
10	7.0	
15	7.0	2.5
17	7.5	5
18	7.0	2.5
19	7.0	5
20	7.0	

The incubation time was 20 hours The final yield was 10.2 g dry weight per litre

each weighing 8 to 12 g. The animals were observed for one week after injection but since most deaths occurred within 3 days, the median lethal dose was calculated on the deaths within this period. Calculation of LD_{50} and its confidence limits was performed according to *Irwin & Cheeseman* (1939). Virulence was determined by a similar procedure. Tenfold dilutions in 0.01 M phosphate buffer were made immediately after sampling. LD_{50} was calculated on the basis of the deaths occurring within 10 days. Since the median lethal dose is about 50 cells per mouse, the actual dilutions used for injection could be spread on nutrient agar plates and viable counts performed.

RESULTS

For studies of growth conditions in batch culture, and the influence of those conditions on the yield and toxicity of the cells, 3-litre cultures were grown in different media and under different aeration conditions (Table 2). The growth vessels used are the two indicated by the numbers 1 and 2 in Table 1. Samples were taken both in the logarithmic phase of growth and in the stationary phase. It was not possible to observe any significant influence on the toxicity of whole cells, killed by exposure to 0.4 per cent formaldehyde, by the different cultivation conditions.

TABLE 2

Toxicity for Mice of Whole Cells of Salmonella typhimurium Killed by Exposure to 0.4 per cent Formaldehyde and Grown in Different Media and under Different Conditions

Medium	Active aeration	Phase of growth	Dry weight of bacteria (g/l)	LD_{50} (mg)
Nutrient broth	+	log	0.47	2.05
	+	stat	0.43	2.28
	–	log	0.35	4
		stat	0.47	2.28
CaY-medium	+	log	0.70	>4
	+	stat	2.64	1.64
	–	log	0.44	3.96
		stat	1.19	2.06
CaYG-medium	+	log	1.09	>8
	+	stat	2.71	1.88
		log	0.83	3.92
		stat	1.53	3.43
H-medium	+	log	0.41	2.34
	+	stat	1.10	1.07
		log	0.35	3.73
		stat	0.49	2.77
F-medium	+	log	0.50	3.22
	+	stat	1.70	1.50
	–	log	0.24	2.47
		stat	0.78	2.68

The median lethal doses (LD_{50}) were within 95 per cent confidence limits except for the logarithmic aerated casamino acid-yeast extract media.

Virulence tests were also performed on bacteria grown in different media and sampled from the logarithmic phase as well as from the

The elaboration of a synthetic medium for high yields presents certain problems. If all nutrients are supplied from the beginning, growth may be prevented by high osmotic pressure. The development of an adverse pH may also cause growth inhibition. Therefore the oxidizable substrate may have to be added gradually. Another possibility in overcoming these difficulties is to employ continuous culture. Here the concentrated nutrient solution is continuously added to the growing culture and the concentration of the nutrients is kept at low levels by the consumption of the growing cells.

A report on the biological activities of fractions derived from cells grown under the cultivation conditions elaborated will be published later.

SUMMARY

Toxicity of whole cells of Salmonella typhimurium killed by exposure to formaldehyde was determined in cells grown in different media and under different cultivation conditions. Similarly the virulence to mice was investigated. No significant difference could be noted in virulence or toxicity between cells grown in simple synthetic media and cells grown in complex media nor could any significant difference in these respects be observed between cells from the logarithmic phase of growth and cells from the stationary phase. Nor did increased aeration efficiency influence significantly the toxicity of the cells.

In cultures of Salmonella typhimurium a considerable increase in the yield of cellular material in simple synthetic media could be achieved by using appropriate aeration and pH control. In a medium containing glucose, lactate and inorganic salts a yield of 10.2 g dry weight of cells per litre (corresponding to a population density of about 10^{11} cells per ml) was obtained in 2 litre shake cultures with active aeration.

A simple continuous culture apparatus for culture volumes up to one litre has been developed and used for the propagation of Salmonella typhimurium cells.

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Several continuous cultures were run, employing different aeration equipments. The culture volume was 400 ml. Gas distribution cylinders were found to provide good mixing and aeration of the cultures (cf. *Table 1*). The amount of air introduced was not allowed to exceed 1 litre per minute; if larger amounts were introduced, foaming occurred.

Runs of 7–10 days were performed in the equipment studies. The F-medium was employed, the ammonium chloride concentration was however 400 mg per litre instead of 1000 mg per litre. The yield obtained in the steady state was then 1.2 g dry weight of bacterial cells per litre of culture, representing the maximum yield obtainable in this medium when the nitrogen source is completely exhausted. No ammonia could be detected by the Nessler method, in the supernatant after centrifugation of samples from the culture. The rate of addition of fresh medium to the cultures, the flow rate, was kept between 100 and 150 ml per hour in these experiments.

DISCUSSION

Fractionation of pathogenic bacteria for the study of the biological activities of different cell components, calls for relatively large amounts of cellular material. The aim of the present investigation was to elaborate cultivation techniques for the preparation of Salmonella typhimurium cells in submerged culture in quantities required for this type of work. It is a well-known fact that many bacteria belonging to the Enterobacteriaceae family grow readily in simple synthetic media containing an organic carbon and energy source, an ammonium salt as the sole nitrogen source, phosphate, sulphate and a series of metal ions supplied as inorganic salts. These simple "synthetic" media offer great advantages in cultivation techniques as compared to complex media, as for example nutrient broth. High yields will easily be obtained in the synthetic media by increasing the aeration efficiency by means of active aeration in the cultures (*Smith & Johnson* 1954).

As an indication of the biological activities of bacteria grown in different media and under different cultivation conditions, virulence and toxicity tests on mice were performed. Since no significant differences in these respects were observed as a result of the changed environmental conditions, it was decided to develop the cultivation methods on basis of a simple synthetic medium.

Simple synthetic media have been used successfully by earlier workers for the preparation of immunogenic substances (*Freeman et al.* 1940, *Kovaleva* 1959, *Ribi et al.* 1959). The limited use of synthetic media for the cultivation of cells for immunological studies and for the preparation of vaccines may be ascribed to the fact that low yields are obtained when conventional cultivation procedures are employed.

FROM THE DEPARTMENT OF BACTERIOLOGY KAROLINSKA INSTITUTET STOCKHOLM (HEAD PROF BERNDT MALMGREN M D)

PREPARATION OF BIOLOGICALLY ACTIVE FRACTIONS FROM SALMONELLA TYPHIMURIUM

2 *Disintegration of Pathogenic Micro Organisms*

By

I Edebo and T Holme

Received 5 ix 60

By pressing micro organisms in the frozen state it is possible to obtain a relatively extensive degree of disintegration Satisfactory disintegration is provided by the Hughes press when operated at about —20° C where the frozen bacteria are forced through a very narrow slit which is formed by clamping together two metal blocks (*Hughes* 1951) The material from the disintegrated micro organisms is however somewhat difficult to collect from the Hughes press and for this reason pathogens cannot be operated in this press without hazards or special precautions being taken Another press called the X press was developed by *Edebo* (1960) In this press the frozen material is pressed through a canal (length 10 mm diam 1 5 5 mm) at 2[illegible] C The pressure needed for forcing the material through the canal is about 2 000 atm The material is facilely collected as a frozen cylinder Another advantage of this press is that the microorganisms can be pressed several times without dismantling the apparatus After one passage up to 10 per cent of unbroken cells often remains as shown by phase contrast microscopy An increased number of passages results in a decrease in the number of unbroken cells and also gives a further disintegration of the cellular constituents as revealed by examination of the cell walls (*Edebo* 1960) In an attempt to obtain a freeze pressing technique suitable for pathogens the press described below was developed In this press it is possible to treat one sample of 10 g wet weight by three passages or to treat three samples once in a procedure lasting not more than ten minutes

MATERIALS AND METHODS

Construction of the Press (See Fig 1)

The main block is made of stainless steel (UHB Stainless 44 Rockwell C 22 6 Hv 45 Brinell 245) and contains three pressing chambers (A B and C) Chambers A

We are much indebted to Miss *Mareike Darson* for her aid in the electron microscopial work and to Mr *Anders Bglén* for skilful workshop assistance

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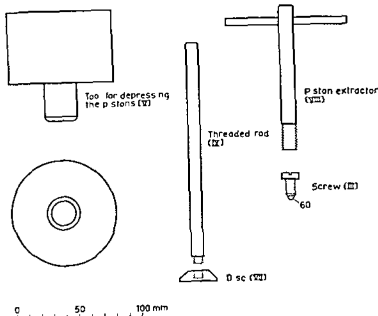


Fig 1 b

efficient scal ng by the screws the bottom of the hole was given an angle of about 1°0 and the t p of the screws an angle of 60°

The three pistons (IV) and the t ol (V) for depressing the p stons are made from hardened tool steel (UHB Arne R ckwell C 60 5 Hv 724) The pistons are chromium plated in order to increase the hardness of the surface and to prevent rusting They are suppl ed with a furrow to collect the material which might sl p between the piston and the wall f the chamber The cl arance between the pistons and the chambers is 0 03 0 04 mm

The apparatus s also provi led with a lock ng mechanism (VI) for the pistons which consists of a sector with a shaft to retain the pistons in depressed position

The pressure is applie l with the aid of a hydraul c jack The pressure required t f rce the material thr ugh the canals is dependent on the temperature in a manner which ind cates that phase transitions of ice (*Tammann* 1903 *Bridgman* 1912 a p r cess wh ch has a sl ght disintegrating effect in itself (*Edebo & Heden* 1960 are also involve l in th s p r cedure

Operat of the Press

After harv
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when the material fl ws into a part cular hole and as rinsers of the canals when the pi t ns are approaching the bottoms of the holes thus reducing the loss of material

First the piston A is presse l down int hole A and the material flows into hole B

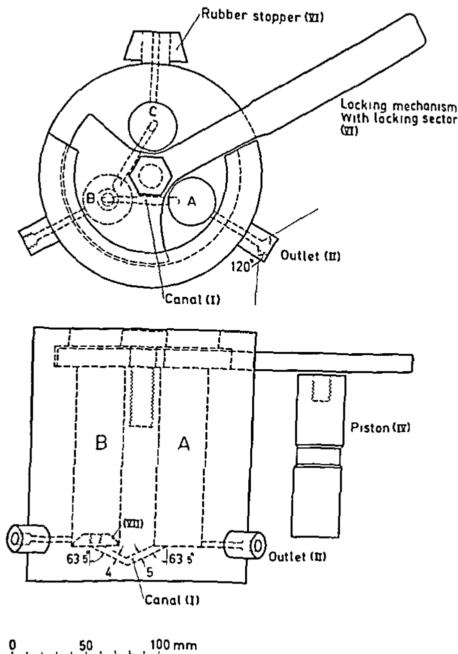


Fig 1a

and B are connected by a 4 5 mm wide canal (I) inclined at an angle of 127°. chambers B and C are similarly joined The canals are made by drilling from below from two directions and filling in the parts below the intersection The angle of 63 5° ($= \frac{127°}{2}$) was chosen as the angle (α) is then relatively large giving the canal

only ... is relatively small and thus the length
of th ... xactly 63 5° is due to the fact that tan
63 5 ... on when the canals were drilled as it
was ... ersection Each chamber has a direct
outlet (II) the outlets from A and B can be closed by screws (III) In order to obtain

Piston A is then locked in this position by the locking sector and piston B is forced down The material then flows into hole C The locking sector is then turned to seal piston B If this is difficult because of reversal pressure by piston A on the sector the latter can be loosened by depressing the sector not more than one millimeter Piston C is then pressed down into hole C and the material is extruded through the outflow The outflow canal is lengthened through a knob (II) welded on to the main body to allow a sterile connection to a thick walled suction flask which is sealed with a perforated rubber stopper (VI) The use of a brittle collecting jar is inadvisable as it might not withstand the sudden outflow of the material

When pressing three samples once each a disc (VII) is placed at the bottom of hole B in order to seal off the communications between the different holes the press is cooled and the material (3 × 50 g) filled into each hole In order to reduce the loss of material an ice plunger may be cast on the top of each cell sample No screws are now placed in the outlets First piston B is pressed down into hole B and locked in position by the sector after which the other pistons are pressed down

by a threaded rod (IX) The screws (when pressing one sample) are removed to facilitate the cleaning of the canals

RESULTS

The press has been used mostly for disintegration of Salmonella typhimurium One pressing causes a disintegration of about 95 per cent (Fig 2) and three pressings a disintegration of more than 99 per cent of the cells (Fig 3) No leakage has been observed around the pistons or around the screws The ice plungers should however be cast carefully to avoid splitting when the pressed material flows out

The pressed material has been used in an investigation of the biological activity of different components of Salmonella typhimurium and reproducible results have been obtained

It was also found here that three pressings disintegrated the cell walls into smaller units (Figs 2 and 3)

DISCUSSION

Many disintegration methods for micro organisms are now available most of them developed for biochemical investigations of non pathogenic micro organisms Some of them may be adaptable for work with pathogenic micro organisms Recently the principle of disintegration by extrusion through a small orifice originally used in the French cell press (*Milner et al* 1950 *French et al* 1955) was adapted for work with pathogenic bacteria and cell wall preparations have been prepared from Mycobacterium tuberculosis (*Ribi et al* 1959) The press described here is constructed to make the freeze pressing techniques (*Hughes* 1951 *Edebo* 1960) applicable to the disintegration of pathogenic micro organisms

When the press is operated at 10 to 12° C, pressures of about 1000 atm can be used to force the material through the canals, and the

Fig. 2
Salmonella typhimurium pressed once. The micrograph shows cell walls and fragments of cell walls × 24 000

Fig. 3
Salmonella typhimurium pressed three times. Only fragments of cell walls are observed × 24 000

FROM THE DEPARTMENT OF BACTERIOLOGY, KAROLINSKA INSTITUTET, STOCKHOLM (HEAD: PROF. BERNDT MALMGREN, M.D.)

DEMONSTRATION OF INTRACELLULAR POLYSACCHARIDE IN ESCHERICHIA COLI BY ELECTRON MICROSCOPY AND BY CYTOCHEMICAL METHODS

By

T. HOLME and B. CEDERGREN

Received 5. ix. 60

Earlier investigations on polysaccharide synthesis in Escherichia coli B have shown that a polysaccharide identified as glycogen accumulates in this organism under conditions of nitrogen starvation (*Holme & Palmstierna* 1956). The main fraction of the glycogen accumulated has a particle size of about 75 mμ, as determined by light scattering on glycogen isolated by alkaline hydrolysis of the bacterial cells followed by precipitation with ethanol (*Holme, Laurent & Palmstierna* 1957).

Electron micrographs of ultra thin sections of glycogenrich cells showed that the cytoplasm of these cells was loaded with granules having a diameter of 50-100 mμ. Similar granules were only [illegible] granules scattered throughout the whole cytoplasm but since the nuclear material occupies the central part of the cell, the granules are predominantly located at the ends.

It has frequently been observed that staining for polysaccharides in bacteria belonging to the Enterobacteriaceae family reveals a bipolar arrangement of the stained material (*Lankford et al.* 1951, *Duguid & Wilkinson* 1953).

In the present paper an attempt has been made to correlate more closely the electron microscopical findings to the cytochemical picture. The periodic acid Schiff reaction (*Hotchkiss* 1948, *McManus* 1948) has been performed on cells with different content of glycogen. Different fixation procedures have been tested and electron microscopy has been used for the control of the effects of the fixation and the staining procedures on the localization of the glycogen in the bacterial cells.

Our thanks are due to Mrs. *Karin Persson* and Miss *Kerstin Lindstedt* for their skilled technical assistance.

disintegrated material is then extruded rather slowly and is readily collected Salmonella typhimurium is easily disintegrated at this temperature When more resistant cells are to be treated, operation at —25° C might be advantageous for the disintegration Pressing at —25° C involves phase changes from ice I (ordinary ice) to ice III (*Tammann 1903*, *Bridgman* 1912) during the flow of the material This is known to be accompanied by disintegration of a great number of different microorganisms (*Edebo* 1960) Pressing at —10 to —12° C involves phase changes between ice I and liquid When the press described here is operated at —25° C, an almost explosive outflow of material often occurs which occasionally cracks the collecting flask For safety reasons, at temperatures below —10 to —12° C, it is therefore recommended that steel-containers threaded on the outlets be used for the collection of the disintegrated material, and, when cells are pressed three times, stell discs should be placed above the ice plungers

So far, the press has been tested on a limited number of different micro-organisms However, it is reasonable to suppose that the mechanism of disintegration in this press is similar to that of Hughes' press and the X-press both of which have been applied to a wide range of different micro-organisms, and have been shown to give both a good disintegration effect and preparations which are biologically highly active

SUMMARY

A disintegration method for pathogenic micro-organisms has been developed The micro-organisms are pressed in the frozen state at about —12° C and 1000 atm through canals 4 to 5 mm wide The material need not be thawed during the whole disintegration procedure Preparations with good biological activity have been obtained

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cent ethanol 70 per cent ethanol, ethanol formaldehyde, (in final concentrations of 70 and 10 per cent, respectively), ethanol acetic acid (3 + 1), and ethanol acetic acid chloroform (6 + 1 + 3)

TABLE 1

Strain	Medium	Time after inoculation in hours	Number of cells per ml × 10^{-8}	Dry weight of cells per litre of culture (mg)	Dry weight per cells (mg × 10^{-10})	Glycogen per cent of dry weight
I 77a	Unlimited	0	2 0	–	–	–
		4 5	14	–	–	–
		5 5	21	374	1 8	2 8
		8	24	434	1 8	2 4
	N defic	0	2 0	–	–	–
		3 5	11	202	1 8	4 0
		6	27	426	1 6	15 6
		8	28	463	1 7	25 0
		12	30	518	1 7	29 4
B	Unlimited	0	2 0	–	–	–
		4	8 7	–	–	–
		6	20	237	1 2	<1
		8	40	408	1 0	1 6
	N defic	0	1 0	–	–	
		4	4 9	36	1 1	2 7
		6	12	387	1 5	1 7
		8	22	374	1 7	10 0

The electron micrographs showed in accordance with earlier findings, a large number of "holes" in the cytoplasm *of glycogen-rich cells* A good correlation between the number of these "holes" and the glycogen content of the cells was noted (Figs 2–3)

An electron micrograph was also made from a section of cells taken from a sample which had been treated with the complete PAS staining procedure (Fig 4) The cells were fixed in 70 per cent ethanol before staining The localization of the glycogen appears not to have been changed to any appreciable extent by this treatment when comparison is made with the untreated cells

The method of Themann (1960) was also employed (Fig 5) This method includes treating the cells with Best's carmine solution before embedding them *for sectioning* Electron dense granules, of about the same size and location as the "holes" observed in cells treated according to the conventional procedure, *could be seen in the* cytoplasm In this case also, a good correlation between the number of granules and the glycogen content of the cells could be noted

DISCUSSION

In an earlier study the occurrence of a large number of "holes" in the cytoplasm could be noted in electron micrographs of ultra thin sections of E. coli cells having a high content of glycogen (*Cedergren & Holme*

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cent ethanol, 70 per cent ethanol, ethanol-formaldehyde, (in final concentrations of 70 and 10 per cent, respectively), ethanol acetic acid (3 + 1), and ethanol acetic acid chloroform (6 + 1 + 3)

TABLE 1

Strain	Medium	Time after inoculation in hours	Number of cells per ml × 10⁻⁸	Dry weight of cells per litre of culture (mg)	Dry weight per cells (mg × 10⁻¹⁰)	Glycogen per cent of dry weight
E 77a	Unlimited	0	2 0	–	–	–
		4 5	14	–	–	–
		5 5	21	374	1 8	2 8
		8	24	434	1 8	2 4
	N defic	0	2 0	–	–	–
		3 5	11	202	1 8	4 0
		6	27	426	1 6	15 6
		8	28	469	1 7	25 0
		12	30	518	1 7	29 4
B	Unlimited	0	2 0	–	–	–
		4	8 7	–	–	–
		6	20	237	1 2	<1
		8	40	408	1 0	1 6
	N defic	0	1 0	–	–	–
		4	4 9	56	1 1	2 7
		6	12	187	1 5	1 7
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MATERIALS AND METHODS

Strains Two strains of Escherichia coli have been used, strain B and 177a the latter being a type strain obtained from Statens Seruminstitut, Copenhagen[1] In a screening test the latter strain was found to accumulate glycogen to an exceptionally high extent

Media A synthetic medium was used (*Friedlein* 1928) employing sodium lactate as the carbon source and ammonium chloride as the sole nitrogen source In media used for the preparation of nitrogen starved cultures the ammonium chloride concentration was 3.7×10^{-3} M In order to obtain cells in the logarithmic phase of growth, the bacteria were cultivated in unlimited medium

Cultivation Cultures were grown in 6 litres Erlenmeyer flasks under active aeration on a rotary shaker in a constant temperature room at 37° C

Polysaccharide determinations For the estimation of the glycogen content the cells were subjected to hydrolysis for one hour in 30 per cent potassium hydroxide followed by precipitation with ethanol A slight modification of the anthrone method described by *Snell & Snell* (1953) was used for the determination of the polysaccharide in the precipitate

Periodic acid Schiff reaction Samples from the liquid cultures were cooled rapidly and centrifuged A suitable amount of material was then spread on a slide and allowed to dry Fixation was performed immediately after drying The periodic acid Schiff (PAS) staining was performed according to *Glick* (1949) The periodic acid was dissolved in 70 per cent ethanol Three smears were made from each sample the first was used for the complete PAS reaction the second was used as a control where the periodic acid treatment was omitted No staining of the cells was ever noted in the control smears The third smear was stained with carbol fuchsin according to Ziehl diluted 1 10 This staining was included to make it possible to study the size and shape of the whole cell which was particularly important when using cells with a very low glycogen content which did not show any visible PAS reaction

Electron microscopical techniques Two different procedures were applied for the preparation of cells for electron microscopy .
tetroxide fixation followed by the embedd
(1958) Ethanol was used as dehydrating
Epon[2] were used as embedding media See
Themann (1960) Also in this case Araldite
Sectioning was performed with a LKB 4800 Ultrotome The microscope used was a RCA EMU 3 A

RESULTS

Two cultures were grown of each of the two strains used, one in unlimited medium to obtain logarithmically growing cells and one in nitrogen-deficient medium to obtain starved cells rich in glycogen The bacterial counts, dry weight and glycogen determinations are presented in Table 1

In the PAS-stained smears a good correlation was noted between the staining intensity of the cells and their glycogen content (Fig 1)

A pronounced bipolar staining of the cells was also observed in all PAS-treated smears (Fig 1) Different fixation procedures were tested for their influence on the localization of the stained material in the bacterial cells No difference in the staining properties of the cells could be noted with the following fixatives osmium vapor 100 per

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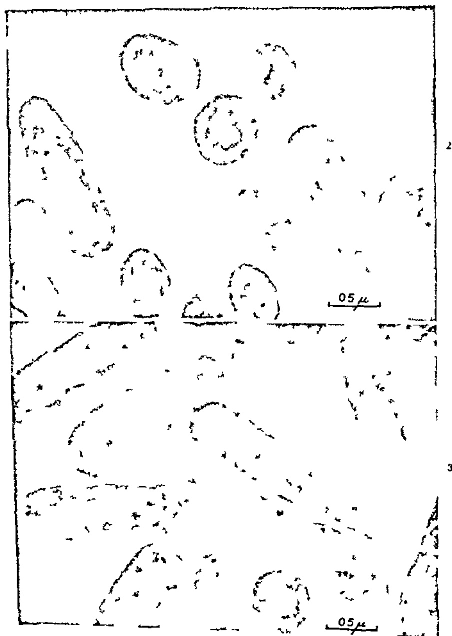
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Fig 1

Periodic acid Schiff-stained E coli cells with different content of glycogen (1600 ×)
a d Strain E77a — *a* Nitrogen starved cells, glycogen content 15 6 per cent — *b* Nitrogen starved cells, glycogen content 25 0 per cent — *c* Nitrogen starved cells, glycogen content 29 4 per cent — *d* Cells from the logarithmic phase in unlimited medium, glycogen content 2 8 per cent
e f Strain B — *e* Nitrogen starved cells glycogen content 10 0 per cent — *f* Cells from the logarithmic phase in unlimited medium glycogen content < 1 per cent

1959) Such "holes" could only occasionally be found in cells with a low glycogen content These findings indicated that glycogen accumulates in this organism in the form of submicroscopic granules In support of this view a good correlation was always noted between the number of "holes" and the glycogen content of the cells Furthermore, a good agreement could be shown to exist between the diameter of the "holes" and the particle size of glycogen as determined by light scattering on glycogen, isolated from the cells by chemical means

SEPARATION OF THE WASSERMANN REAGINS BY CELLULOSE COLUMN CHROMATOGRAPHY OF SERUM

By

ANNA BRITA LAURELL and JORGEN MALMQUIST

Received 5 ix 60

It has been shown by serological as well as by physico-chemical methods that the Wassermann reagins are heterogeneous (*Davis et al* 1945 *Neurath et al* 1947 *Schmidt* 1955 *Laurell* 1955 *Laurell & Lindau* 1958)

In the present investigation the Wassermann reagins in different types of sera have been studied with the aid of column chromatography on DEAE cellulose

MATERIAL

The material consisted of sera from 8 patients with syphilis (primary syphilis in 2 secondary in 2 and tertiary in 4 6 patients with various diseases showing biologic false p t e Wassermann reactions and 2 healthy persons

METHODS

Column chr matography on DEAE (d ethylam noethyl ... lo ...

... a t e ... 5 5 ml f 0 01 m pH 8 0 sod um phosphate buffer and a t rr ng levice The fla k c nta n ng the s d um phosphate buffer was nnecte l by s phon tubing to the ther flask as w ll as to the column tube After the gra l ent elut on was complete and the *flasks* were empt ed 100 ml of 0 3 M m n sod um ph phate was p ured int the st rr ng flask and allowed to pass through the c l mn Th step was repeated w th a second s m lar ... ph phate In h way ...

... was leterm ned by measur ng the pt cal den ty at 280 mμ in a Beckman ...

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FROM THE BACTERIOLOGICAL INSTITUTE, LUND SWEDEN
HEAD PROFESSOR RUNE GRUBB

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In the present investigation the Wassermann reagins in different types of sera have been studied with the aid of column chromatography on DEAE-cellulose

MATERIAL

The material consisted of sera from 8 patients with syphilis (primary syphilis in 2 secondary in 2 and tertiary in 4), 6 patients with various diseases showing biologic false positive Wassermann reactions, and 2 healthy persons

METHODS

Column chromatography on DEAE [illegible]
by a method differing s[illegible]
(10 8 The [illegible]

[illegible] flask as well as to the column tube After the gradient elution was complete and the flasks were emptied 100 ml of 0 3 M monosodium phosphate was poured into the stirring flask and allowed to pass through the column This step was repeated with a second similar quantity of the phosphate In this way [illegible]

[illegible] *Protein concentration* in each fraction was determined by measuring the optical density at 280 mμ in a Beckman DU [illegible]

Immuno electrophoresis [illegible]
were pooled and concentra[illegible]
phoretic analysis which was [illegible]

Wassermann reaction Fa[illegible] the routine method used in this institute employing cardiolipin and a crude antigen

The tubes corresponding to peaks IV and V were dialyzed against 0.9 per cent saline in the cold over night before testing because the high phosphate concentration in these fractions would otherwise interfere with the complement fixation test.

RESULTS

A typical elution diagram is shown in Fig. 1.

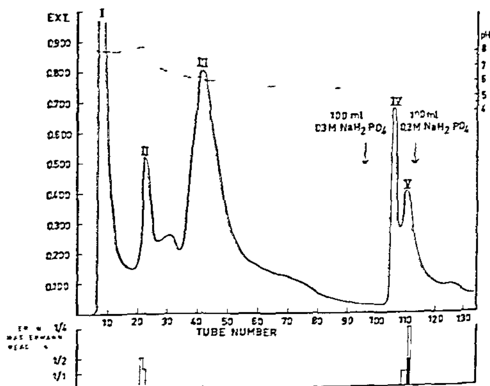


Fig. 1.
Effluent diagram of tertiary syphilitic serum (C9). The titers of the Wassermann positive fractions are shown below the diagram.

▮ titer with crude antigen. ▯ titer with cardiolipin.

On analysis with immuno-electrophoresis of the 2–3 tubes corresponding to summit of peak I only gamma globulin was detected. Immuno-electrophoresis of peak II revealed not only transferrin but also gamma globulin with an intermediate electrophoretic migration rate. Peak III was not analyzed by immuno-electrophoresis. However it seems reasonable to assume that it contained the same components as the third peak of Fahey et al., namely albumin, alpha globulins and beta globulins. The fractions of peak IV contained, when analyzed by immuno-electrophoresis, a mixture of albumin, alpha globulins and beta globulins and possibly represented traces of the substances in the third peak. Peak V was shown to contain the beta 2 M fraction according to Burtin et al. (1957) (19 S γ_1 globulin according to Muller-Eberhard et al. 1956) and a component of the alpha globulins.

Investigation in the Wassermann Reaction

Table 1 shows the location of fractions yielding positive Wassermann reaction in the sera studied

TABLE 1

Serum	Pos Wassermann test in peak I	peak II	peak V
Primary syphilitic			
WR 86	+*	-	+
WR 128	+		+
Secondary syphilitic			
WR 87	+		+*
WR 88	+		+
Tertiary syphilitic			
C 9 } §		+	+
C 1 } §		+	+
WR 47 } §		+	+
WR 53 } §		+	+
C 10	+	+	+
WR 137	+	+	+
Biologic false positive			
WR 21		-	+
WR 110			+
WR 131	+		+
WR 136	+		+
WR 140	-		+
C 13			anticompl
Normal			
SB			+
ABI			weakly anticompl *

* positive only after tenfold concentration
§ from the same patient at different sampling occasions

The two sera from cases of primary syphilis showed serologic activity in peak V Serum WR 86 was from a case of early primary syphilis This serum showed activity in the peak I fraction only after the latter had been concentrated tenfold Serum WR 128 was from a case of primary syphilis in a more advanced stage This serum gave a positive Wassermann reaction in the peak I fraction even when not concentrated No activity was found in any of the other fractions of these two sera

Both of the sera from cases of secondary syphilis were active in peak I One of the sera gave a positive reaction also in peak V, while the other did so only after tenfold concentration of the fractions of this peak The other fractions in these two sera were serologically inactive

All the sera from cases of tertiary syphilis gave positive reactions in

the peak *II* fraction in contrast to what was found for patients with primary and secondary syphilis and sera from patients with biologic false positive reactions. The peak V fraction of all the tertiary syphilitic sera was serologically active, while only two of these sera showed positive reaction also in peak I fraction.

Of the 6 biologic false positive sera 3 showed activity only in the peak V fraction, two sera showed activity in both peaks I and V, while one serum was negative in all fractions, but showed an anticomplementary effect in peak V.

Serum WR 21 with a positive Wassermann reaction in the peak V fraction also showed a strong anticomplementary titre in that peak. The Wassermann reagin and the anticomplementary substance could, however, be separated because they occupied different positions within the peak.

Those fractions corresponding to peaks I, II and V of the different sera which gave negative reactions were usually concentrated tenfold by ultrafiltration against 0.9 per cent saline and retested. Only in 2 instances did the previously negative fractions turn positive after concentration (see Table 1).

Two sera from healthy individuals were studied and both were run twice with consistent results. One of the sera (S.B.) gave a positive Wassermann reaction in the peak V fraction. The fractions of the normal serum (A.B.L.) were serologically negative. Tenfold concentration of the peaks of this serum resulted in a weak anticomplementary effect in peak V, while the other peaks remained negative.

DISCUSSION

The observation made previously (*Davis et al.* 1945, *Neurath et al.* 1947, *Laurell* 1955) and in the present investigation of the occurrence of fractions of normal sera giving positive serologic tests for syphilis suggests the possibility that normal sera may contain minute amounts of Wassermann reagins inhibited by other serum components in unfractionated serum. Those fractions of normal sera that are active in the serologic tests for syphilis with lipid antigen show a biologic false positive type of reaction (*Neurath et al.* 1947, *Laurell* 1955).

Judging from the results of studies using ultracentrifugation (*Davis et al.* 1945) and the finding of a positive Wassermann reaction in the beta 2-M containing peak V fraction of one of the present normal sera this normally occuring reagin may be a macroglobulin. In all but one of the pathological sera investigated the peak V fraction regularly showed serologic activity. It seems that most or all sera giving positive serodiagnostic reactions for syphilis contain a Wassermann reagin of macroglobulin type, that might be identical with that present in fractions of normal serum.

It is known that from a serological as well as physico chemical point

of view, the sera from early cases of primary syphilis differ from sera from cases of secondary and late syphilis Thus, in the Neurath euglobulin inhibition test (*Neurath et al* 1947) and in the Kahn verification test (*Kahn* 1940, 1951) sera from early primary syphilis often behave in the same way as biologic false positive sera In addition, the tendency of primary syphilitic sera, like biologic false positive sera, to react with crude lipid antigen earlier and in higher titres than with cardiolipin antigen (*Lundbeck* 1952, *Schmidt* 1952, *Laurell & Lindau* 1958) suggests that the type of Wassermann reagins found in early primary syphilis differ from those found in secondary and late syphilis Judging from the findings made in our small number of cases, this difference might be explained by the assumption that the macroglobulin type of reagin giving a "biologic false positive type" of reaction is predominant in sera from early primary syphilis and that progression of the disease to the secondary and tertiary stages is accompanied by the formation of more low molecular types of reagins belonging to peak I and peak II fractions respectively, these later appearing types of reagins presumably giving a 'syphilitic type of reaction During the tertiary stage of syphilis the activity in peak I apparently may disappear (Table 1)

It is noteworthy that the peak II fraction of all the sera from cases of late syphilis, but of none of the biologic false positive sera, showed serologic activity All of the biologic false positive sera studied had a titer in the Wassermann reaction of the same order of magnitude as the tertiary syphilitic sera Thus differences in concentration might be excluded as the cause of the difference in chromatographic behaviour between these two types of sera This also holds for the comparison of sera from cases of late syphilis with sera from cases of primary and secondary syphilis However, it is quite clear that the number of sera investigated is too small to permit definite distinction of late syphilitic sera from other Wassermann positive sera on the basis of the types of Wassermann reagins found in a given case

The observations presented in this paper suggest the existence of three different types of Wassermann reagins Ultracentrifugation (*Davis et al* 1945) as well as electrophoresis (*Laurell* 1955) showed only two types The difference in the results can be explained by the fact that the chromatographic method permits separation of the 7 S and 19 S gammaglobulins (not distinctly separated by preparative paper electrophoresis) as well as of the 7 S gammaglobulin of peak I and the 7 S gammaglobulin of peak II (not separated by ultracentrifugation)

SUMMARY

(1) Sera from cases of syphilis in different stages, sera giving biologic false positive Wassermann reaction, and normal sera have been fractionated by column chromatography on DEAE-cellulose

(2) Wassermann reagin could be demonstrated in three separate chromatographic fractions, indicating the existence of three individual Wassermann reagins

(3) One of the reagin types was found in all tertiary syphilitic sera but in none of the other sera studied

(4) In one of the two normal sera studied Wassermann reagin was found after fractionation

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HEAD KRISTEN ARNESEN M D

THE HISTOLOGY OF THE SENILE ENDOMETRIUM

An Autopsy Study

By

TORE NOER

Received 11 vii 60

The histology of the senile endometrium has been the subject of astonishingly few systematic studies and it is treated in a rather summary way in most textbooks note being made of its general atrophy the cystic glands and the fibrosis of the endometrial stroma

The studies by *Breipohl* (1935) *Taylor & Millen* (1938) *Keller & Adrian* (1939) *Novak & Richardson* (1941) and *Dhom* (1952) are all based upon series of routine endometrial biopsies Studies by *Foix* (1952) and *McBride* (1954) are based on curettings from presumable normal endometria *McBride* also examined material from postmortems *Gianarelli* (1948) examined uteri from a combined surgical and autopsy material *Speert* (1949) examined uteri removed at operations for genital prolapse

For a systematic study an autopsy material has several advantages In the microscopical slides topographical relationships are preserved All polyps are noted whereas in curetted material they may have been left in the uterus or are fragmented and not recognized An autopsy material is unselected whereas a material from curettings or major operations is heavily biased Uteri removed at operations for genital prolapse usually are from multiparous women

A serious drawback of working with an autopsy material is that postmortal autolysis can make the judgment of the endometrium difficult The surface epithelium is often destroyed and in the glands the epithelium may be desquamated and curled up in the lumen The stromal fibrils loose their distinctness However there are usually representative parts that allow a fair degree of judging

PRESENT MATERIAL

Th[illegible]
fr[illegible]
pa[illegible] [illegible] have passed 55 years in the
cal [illegible] death are included

Nine cases have been excluded from the material because of endometrial adenocarcinoma with total destruction of endometrium in two cases pyometra with

destruction of endometrium in two cases (both of tuberculous origin histologically) chronic tuberculous endometritis in two cases earlier irradiation of the uterus in two cases and earlier estrogen treatment in one case

The material then consists of 129 endometriums The age distribution of the material appears from Table 1 Of the women, 72 had born children 46 were nulliparous and in 11 cases the information is inadequate

The microscopical sections have been stained with haematoxylin eosin and in some cases also with the *van Gieson* and PAS stains

The ovaries have been inspected but slides have not been taken

PRESENT FINDINGS

Glandular Pattern

In reviewing the present material, the author in agreement with *Speert* has arrived at the conviction that atrophy is the dominant feature of the senile endometrium, although in the earlier age groups the atrophy may appear to be in its early beginning, and in some cases there are signs of a certain glandular hyperplasia The general characteristics of the senile endometrium are.

(1) The normal architecture is lost, with its two distinct layers the basalis and the functionalis

(2) A reduction is found in the number of glands, many glands apparently disappearing completely

(3) The glands lose their connection with the surface, and regular surface openings of the glands are seldom seen

(4) *Ectatic and cystic glands are a common finding, this being one* of the main characteristics of the senile endometrium

(5) The epithelial cells of the glands have scant cytoplasm, which makes the nuclei appear situated close together, in different levels in the cells They are round or oval Mitoses are almost never found The cell borders are indistinct

(6) The stromal cells lose their cytoplasm, and consist only of round, dark and small lymphocytelike cells within a finely dispersed fibrillar network Bundles of collagenous fibrils may or may not occur, but a correlation between glandular pattern and degree of fibrosis could not be found Apparently the collagenous fibrils may invade from the myometrium, as they occur mostly in the deeper layer of the endometrium

However, within this general pattern a great deal of variation exists The author thinks the following main types of senile endometrium may be recognized

(1) Quiescent type
(2) Cystic atrophic type *
(3) Hyperplastic trend

It must be realized that diffuse transitions exist between the different groups, and that usually atrophy is predominating

With the aforementioned reservation a description of the appear-

ance of the different types will be given Also some special features of the postmenopausal endometrium will be related

(1) *Quiescent type* In this group are included those endometria which still have preserved the essential traits of the fertile proliferative phase The glands are tubular more or less sparsely scattered with a cubic or slightly cylindric epithelium However mitoses are very few or absent A characteristic finding is the horizontal position of the gland tubules instead of vertical to the surface The stroma is loose some what fibrous Some ectatic glands may occur heralding the impending cystic atrophy (Fig 1)

A picture similar to the fertile secretory phase was never found although PAS positive material in the glands existed

Of the endometriums 10 or 7 8 per cent were classified as quiescent Seven individuals were less than 59 years old and the oldest one was 75 years old

(2) *Cystic atropic type* This pattern is the far most frequent As the name implies the picture is dominated by the reduced thickness of the endometrium and the decreased number of all elements as well as by the cystic glandular lumina (Fig 2) The cysts are grouped to gether or diffusely scattered They may be large with diameters reach ing three mm being visible with the naked eye Usually the cysts are round but their form is often irregular They may contain amorphous or hyaline material which infrequently can be PAS positive

The epithelial cells may be cuboidal or sometimes flat especially in large cysts However the epithelium in quite large cysts may remain distinctly cuboidal or even slightly cylindric (Fig 3)

A further step in the atrophic process is reached when the endo metrium is reduced to a bare surface epithelium and a scant layer of

or less cystic glands (Fig 4)

of the endometrium

[illegible] were included or 76 per cent Of these six were totally atrophic

The 98 cases come in all age groups but it appears from Table I that the relative frequency is greatest in older age groups

(3) *Hyperplastic trend* The designation senile atrophy with hyper plastic trend is not a lucky one However hyperplasia is the tradi tionally used word Proliferative is the name of a phase of the fertile endometrium and estrogen stimulated is not an appropriate descrip tive term Active trend might be an acceptable substitute

The designation used is proper insofar as it conveys an idea of the combination of traits atrophic and hyperplastic that exists

Falconer (1947) lists the following characteristics of endometrial hyperplasia (1) Abundance of glands (2) Crowding of glands (3) Pictures of invagination (4) Dilated and cystic glands (5) Tall epi thelium containing cylindric cells (6) Hyperplasia of glands limited to clearly outlined patches (7) Dilated lumina of vessels

As will be seen some of these criteria are not applicable to the diagnosis of hyperplasia in the senile endometrium Both the cyst formation, which often lends the endometrium a swiss cheese appearance, the dilated vessels and the frequent cellularity and fibrosis of the stroma, will make the judgment difficult In the present study the *combination of the following* criteria have been required before the designation of hyperplastic trend has been given

(1) Increased number and crowding of glands, with irregularity of shape

(2) An epithelium which deviates from the atrophic type, *viz* is cylindric, with greater amount of cytoplasm and with definite cell borders, and with large oblongated nuclei The number of mitoses is low

There is a diffuse transition from the endometria of the cystic atrophic type with dispersed glands with low epithelium, to those where the glands are more irregular and crowded together, with a cylindric epithelium The prominence of hyperplastic traits may also vary in different parts of the endometrium In many of the cases *McBride's* designation of *cystic gland pattern with active elements* is very appropriate, as the picture is not predominantly either atrophic or hyperplastic (Fig 5) In the present paper the designation hyperplastic has been used only when the picture was decidedly deviating from the usual cystic atrophic endometrium

In other cases the hyperplastic tendency was stronger and more dominating, with thicker endometrium and crowded irregularly formed glands (Figs 6 and 7) The hyperplastic trend was never so pronounced that it became identical with a frank hyperplasia of the fertile type

The clinical records of the patients have been reviewed for abnormal sources of estrogen None of the patients had granulosa cell tumours of the ovaries macroscopically

A hyperplastic trend was found in 21 cases, or 16 3 per cent Their *age distribution* appears from Table 1 The oldest patient in this group was 85 years old

The composition of the whole material appears from Table 1 The material has been broken down as to marital status and number of births, and no significant relationship has been found

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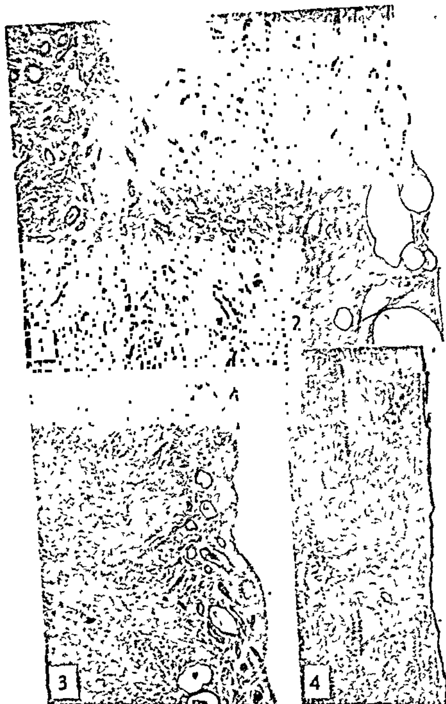
Figs 1 4

Fig 1 Quiescent endometrium in woman aged 60 × 35

Fig 2 Cystic atrophy of the endometrium Age 56 × 35

Fig 3 Cystic atrophy with fibrosis of stroma Age 79 × 35

Fig 4 Total atrophy of the endometrium almost no glands left Age 67 × 35


1
2
3
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A status pseudoangiomatosus was found in 10 uteri, of which nine had teleangiectasies in the endometrium In all there were distinct teleangiectasies in 47 cases, or 35 7 per cent, but in greatly varying degree

Haemorrhages in the endometrium were a very frequent occurrence, and they are often seen macroscopically They usually occur in atrophic endometria, and are frequently combined with a teleangiectatic vascular pattern Only in three cases pigment macrophages were found, although haemolysis of the erythrocytes had occurred more often This may be interpreted to the effect that the bleedings in most cases were recent, and they may be presumed to be agonal A considerable number of patients had received anticoagulation therapy before death

In hyperplastic endometria bleedings were a rare finding

Endometrial Polyps

Polypoid structures emanating from the endometrium were found in 24 cases, or 18 6 per cent In series of autopsies *Speert* found polyps in 17 per cent and *McBride* in 15 per cent In biopsy material the frequency is considerably lower

The polyps are seldom distinctly pedunculated, but have the character of broad-based protuberances of the endometrium, with more or less mushrooming of the edges (Fig 10) Histologically, two main types can be differentiated, designated as polyps with inactive gland pattern, and fibroadenomatous polyps by *McBride* The last type apparently may correspond to *Menge*'s (1922) Korpusadenom der Matrone

The first type corresponds to the common endometrial polyp in fertile age, carrying with it the endometrial cytogenic stroma with some fibrosis, and with glands of distinctly endometrial nature Their glandular picture may run the gamut of different stages of atrophy or proliferation, and in fact some may be indistinguishable from a localized hyperplastic process in the endometrium *Kottmeier* (1950) has put forward criteria for the identification of such polyps in curettings

The second type has as its peculiar characteristic a stroma which is predominantly composed of collagenous fibrils, often with a hyaline degeneration of the matrix The stroma is intermingled between glands which often are markedly cystic, and frequently contain PAS positive material The epithelium does not differ from that of the endometrium, but hyperplastic trends are not seen

This second type of polyps has a close similarity to polyps emanating from the cervical canal or isthmus However, they can be seen based on a definite corporeal endometrium, which often can be seen traversing the base of the fibrous polyp untouched by the fibrosis

The size of the polyps varied between those hardly seen with the naked eye, to polyps filling the whole uterine cavity

In this material there was no significant relation between age or number of children and type or size of polyp Ten of the polyps be-



Figs 8 11

Fig 8 Senile superficial teleangiectasies Age 73 × 89

Fig 9 Status pseudoangiomatosus The picture is from a case of tuberculous pyometra not included in the material Age 77 × 32

Fig 10 Endometrial polyp macroscopically A broad based protuberance on posterior wall of uterine cavity Age 60

Fig 11 Section of common endometrial polyp with carcinomatous growth to the right Age 75 × 26

longed to the first type and 14 to the second In 3 of the 10 polyps of the first type a hyperplastic picture existed, and in one of these a hyperplastic trend existed in the rest of the endometrium Otherwise, the glandular pattern in the polyps and in the rest of the endometrium did *not necessarily correspond*

The three oldest patients were 85 years old The youngest patient with a fibroadenomatous polyp was 59 years

In 15 of the 24 cases of polyps teleangiectasies similar to those of the endometrium were found

None of the polyps found in this investigation had given rise to symptoms that had been noted in the clinical record of the patient Metrorrhagia originating from endometrial polyps certainly is not frequent

Huber (1951) has found a statistically significant higher frequency of genital carcinoma correlated to polyps, although not necessarily in the polyp He thinks the polyps are but an expression of a general proliferative stimulus which can manifest itself also in other parts of the genital system

In this investigation unsuspected adenocarcinoma of the endometrium, in very early stage and without significant invasive growth, was found in two cases In both cases polyps were found, with distinct *malignancy in parts of the polyps As there was also carcinomatous* spreading in the endometrium, the origin from the polyp could not be definitely ascertained

DISCUSSION

The nomenclature and the criteria for classification of the senile endometrium are not universally agreed upon Neither is there agreement upon the relative frequency of the different types

The following grouping is used by *Novak & Richardson* (1) Thin *atrophic mucosa* (2) Moderate proliferation (3) Active hyperplasia (4) Retrogressive hyperplasia *McBride* uses another classification (1) Proliferative pattern (2) Inactive cystic gland pattern (3) Total atrophy (4) Cystic gland pattern with active elements (5) Active hyperplasia *Keller & Adrian* (1939) distinguish between general atrophy, dysplastic atrophy, and atrophy with associated hyperplastic fenomena *Gianaroli* differentiates simple and cystic atrophy *Dhom* and *Kottmeier* (1947) talk of cystic atrophy and various degrees of hyperplasia, and the term "ausgebrannte Hyperplasie is also used

The differentiation of the senile endometrium into subgroups is a purely descriptive one There exists no proof of hormonal or other physiologic mechanisms which justify the differentiation into simple cystic, quiescent, dysplastic and total atrophy, and these terms might possibly be discarded as *superfluous There is not even proof* that they are different stages in a continuous process

That atrophy is the usual picture is agreed upon by most authors The only exceptions to the atrophic state in Speerts 60 cases were three cases of hyperplasia, all of whom had possible abnormal estrogen sources *Foix* finds atrophy in 86 per cent of his cases, of these general atrophy in 20 per cent and cystic atrophy in 80 per cent *McBride* finds atrophy in 65 per cent of a postmortem study However, *Novak & Richardson* found atrophy in less than 50 per cent of their curettings, and *Breipohl* found atrophy in 47 per cent of curettings

Different opinions exist concerning the origin of the cysts *Novak & Richardson* infer that they are remnants of an earlier hyperplasia Their great frequency seems to contradict this In the present material cysts with a diameter of 0 33 mm or more were found in slightly less than two thirds of the cases, and the majority of the rest had ectatic glands, although of smaller caliber *Speert* finds cystic structures in 72 per cent of cases He interprets the cysts as pure retention cysts, and puts importance to the absence of glandular openings on the surface His opinion appears to be the most convincing

The main interest centers on the hyperplastic states in the endometrium This theme is a very complicated one, owing to a confused

cheese pattern, which may not be a genuine hyperplasia at all, but an acyclic proliferative state Then there exists a hyperplasia with more or less irregular overgrowth of glands, adenomatous glandular hyperplasia, "echte Hyperplasie" (*Deelman* 1933) This type appears to be rare At last there comes the question of the inactive, retrogressive or "ausgebrannte" hyperplasia

That frank endometrial cystic hyperplasia exists in old age is known by several publications, and by the fact that it can be produced by the administration of estrogen In series of cases of postmenopausal metrorrhagias various percentages of hyperplasia have been reported *Breipohl* found hyperplasia in 15 of 130 cases, two had granulosa cell tumours of the ovaries *Taylor & Millen* found 11 hyperplasias of 406 cases, four had granulosa cell tumours *Husslein* (1948) found 40 of 200 cases, *Dhom* 420 of 1000 cases, *Sutherland & McBride* (1954) 126 of 1000 of whom 95 were active and 31 inactive hyperplasias *Novak & Richardson* found active hyperplasia in 28 of 137 cases, and retrogressive in 33

In series of postmenopausal, symptomless endometria, hyperplastic states of the endometrium are reported in 1 8 per cent of 1521 patients by *McBride*, and in 12 of 100 cases by *Keller & Adrian* Neither *Gianaroli* nor *Speert* could find any cases of hyperplasia, except where abnormal sources of estrogen existed These reports are not directly commensurable owing to different selection of material and diagnostic criteria

Both *McBride* and *Dhom* notes that the postmenopausal hyperplastic state predominantly occurs in the first years after cessation of menstruation

There is no proof of the actual existence of the mechanism inferred by the names retrogressive and "ausgebrannte" hyperplasia The present author prefers the term hyperplastic trend, which he finds in 22 cases, or 16 per cent

The physiologic background for the postmenopausal endometrial histology is the warrant of this terminology

The senile endometrium has no function, and consequently would be supposed to atrophy, in accordance with the general laws of biology However, the endometrium has its specific physiologic stimuli, *viz* estrogen and the corpus luteum hormone. After the menopause progesterone disappears from the urine, whereas small amounts of estrogen are found, supposedly originating from the suprarenal gland, although there seems to be some activity in the ovary in the first years after the menopause

The postmenopausal endometrium thus is under a continuous, weak estrogenic influence, and is at the same time the subject of an atrophic process Which of these processes wil be the strongest, depends upon the degree of senile involution in the individual, the amount of estrogen produced in the organism, and possibly on other factors

The conclusion is that no theoretical objections exist to the presence of a latent hyperplastic state in the senile endometrium, nor to the rare existence of active hyperplasia without exogenous estrogenic sources

There is no necessity for the hyperplasia to result in metrorrhagia In fact *Novak* (1952) in his textbook holds that the bleeding occurs only after withdrawal of estrogen

SUMMARY

(1) A histological study of an autopsy material consisting of 129 presumably normal postmenopausal endometria is reported

(2) The main factor in forming the histology of the postmenopausal endometrium is atrophy, which is characterized by a loss of fertile endometrial architecture, reduction in number of glands, loss of glandular surface openings, occurrence of ectatic and cystic glands, and changes in the appearance of epithelial and stromal cells with scarcity of cytoplasm The cystic pattern is a very common one, and can not be taken as signifying the existence of a swiss cheese hyperplasia

(3) The author distinguishes three main types of senile endometria, *viz* (a) quiescent, (b) cystic atrophic, and (c) hyperplastic trend

The quiescent endometrium is histologically fairly identical with a fertile, proliferative phase

The cystic atrophic is the common picture

The hyperplastic trend is the designation of those endometria where

the glandular and epithelial pattern deviates from the pure atrophic type giving the endometrium traits similar to those recognized as hyperplastic

(4) A quiescent endometrium was found in 7 8 per cent, a cystic atrophic in 76 per cent, and a hyperplastic trend in 16 3 per cent

(5) Polyps were found in 18 6 per cent They were of two histologic types Unsuspected endometrial adenocarcinoma was found in two of the cases with polyps

(6) The vascular pattern in the postmenopausal endometrium has some features different from that of the fertile

(7) Endometritis of different degrees was found in 12 cases, or 9 3 per cent

(8) There is no theoretical objection to the existence of an asymptomatic hyperplastic trend in the postmenopausal endometrium

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Farkas et al (22) found that a medial calcification ordinarily takes place after the age of twenty, and far more frequently in the aorta than in the peripheral vessels He presumed that the medial calcification was a "pre-stage" to actual atherosclerosis

Blumenthal et al (8) by histological methods demonstrated that a medial calcification always precedes a formation of intimal plaques The relationship between the medial calcification and age was demonstrated on a large material The localization of calcium in the media was not discussed in details Also *Lansing* (43) and his collaborators have described the medial calcification—the elastocalcinosis—as being essential to the formation of the intimal plaques

Levene & López Suarez's (47) publication created an interest in the aortic ground substance and its biochemistry Shortly after several histological studies (65, 67, 77) were published, all describing an increasing accumulation of ground substance in the aorta with age They held that the vascular ground substance has a certain "affinity" to lipoid and calcium Later studies by *Moon & Rinehart* (52), *Rinehart* (56), and *Rinehart & Greenberg* (57) seem to indicate that acid mucopolysaccharides play a certain role in human as well as in experimental atherosclerosis

When examining human aortic material one should keep in mind that not all alterations of the vascular wall are necessarily sclerotic alterations they may be "normal age-alterations", which do not participate in the actual atherosclerotic process

In the present study, which solely deals with age alterations in macroscopically normal aortic tissue and tissue from the pulmonary artery, it is attempted by histochemical methods to determine a possible relationship between lipoid deposit and calcification in elastic vascular tissue and their mutual relations to ground substance and fibrils

MATERIALS AND METHODS

The material consisting of human vascular tissue from 114 individuals in the age group 0 to 90 years was obtained at autopsy partly at the Rigshospital and partly at the Copenhagen County Hospital Gentofte

Tissue was collected from both macroscopically normal aortae and from [illegible]

ar [illegible]
cha [illegible]
pa [illegible]

Furthermore serial specimens from both the thoracic and the abdominal part were taken from macroscopically normal aortae and from normal parts of atherosclerotic aortae The intention was partly *to find* out the variations of the microscopical picture within the individual aorta and partly to [illegible] whether age alterations differ [illegible]

In 32 cases spe[illegible] proximal part an[illegible] oth from the

In order to sup[illegible] material we examined the aorta and the pulmonary artery partly from a patient with *Muller Harbitz's* (54) syndrome and partly from a patient with hypertension in the pulmonary circulation

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ALTERATIONS IN HUMAN AORTA AND PULMONARY ARTERY WITH AGE

By

Sv Bertelsen

Received 2 viii 60

Since *Vogel* (71) in 1847, and later *Virchow* (69), described the accumulation of lipoid in the aorta and its influence on the connective tissue in the vascular wall, several theories have been advanced, attempting to explain the occurrence of lipoid in the aorta, and its relation to the sclerosis and calcification Virchow held that the lipoid deposit in the intima was a degenerative change (metamorphosis), secondary to inflammatory processes in the connective tissue

A few years later *Langhans* (40) described the thickening of the intima, which appears even at an early age He shared Virchow's opinion that a relation could be detected between the lipoid degeneration of the vascular wall and the deposit of calcium In the years that followed not only Langhans but also *Cohnheim* (14), *Lange* (39), *Klotz* (38), and *Aschoff* (3) supported Virchow's theory

By histological examination of a great number of aortae *Ribbert* (55) found that the thickening of the intima starts during infancy and becomes sclerotic in time The accumulation of lipoid in the intima takes place concurrently with—and independent of—the changes of the connective tissue

Leary's (46) *theory that the lipoid is carried with the blood* stream to the intima in large macrophages, was supported by *Gordon* (30) and other investigators The lipoid which gradually appears extracellularly, stimulates to formation of connective tissue and sclerosis

As early as 1905, *Klotz* (38) showed that early in life calcium appears in the media in close relation to the muscle fibres, and that the deposit of calcium is preceded by a lipoid degeneration Klotz further found that the calcium of the media may "invade" into the intima

A few years later *Faber* (19) in a histological study showed that the medial calcification appears *as frequently in large central vessels* as in small peripheral ones He held that the calcification is closely related to the elastic tissue, and that there is no connection between lipoid degeneration and calcification of the vascular wall

Aided by grant from the Danish State Science Foundation

As to the methods of combined PAS Alcian blue staining toluidine blue staining and the different enzymic specificities see a previous publication (*Bertelsen & Jensen* (6))

RESULTS

Aorta

Media

Already from birth the ground substance is increasing constantly with age The elastic fibres, located in the young aorta in a regular, undulated course, are gradually ruptured from each other by the Alcian blue positive and strongly metachromatic ground substance Simultaneously, an increase takes place in the number of cells in the ground substance, the majority of these cells are fibroblasts (Fig 1) The accumulation of ground substance is particularly strong in the innermost half of the media

The growth of the ground substance involves a fragmentation of the elastic fibres, and their course is becoming irregular, often duplicated Numerous communicants are visible between the individual fibres (Figs 1, 2)

During the first 10 15 years acid mucopolysaccharides in particular are deposited among the elastic fibrils but gradually a substance appears which reacts strongly positive to Schiff s reagent after oxidation with periodic acid This deposit, often located close to the elastic fibrils, will—in fairly young aortae—by staining with silver and van Gieson Hansen s stain, appear to consist largely of reticular fibres (Fig 3) and fragments of fine collagen like fibrils With increasing years we find besides these fibrillar structures, an accumulation of PAS positive substance composed of glycoproteins

This accumulation of glycoproteins is most evident in the intimal half of the media, where fibroblast like cells surrounded by spots of PAS positive substance or cells with PAS positive cytoplasma, can often be demonstrated This increase in the accumulation of glycoproteins takes place particularly after the medial mineralization has started (Fig 9)

The reaction of the few smooth muscle cells, located in the aortic wall is also faintly PAS positive

The collagen fragments observable in van Gieson Hansen s staining, are increasing with age both in length and in number Throughout the ground substance these fragments make their appearance among the elastic fibres (Fig 10) As just mentioned some *argyrophile fibres*, located along the elastic fibrils are visible in the silver stained preparation In ageing this phenomenon too, seems to become more pronounced (Fig 3)

The content of lipoid in the media is scanty, especially in relation to the accumulation, in the intima Lipoid deposits in the ground substance are observed only in the luminal third of the media, and then only when large amounts are present in the intima (Fig 11)

The patient with Muller-Harbitz's syndrome was a 52 year old man whose brother suffered from a similar disease. During 10-15 years he had had hypercholesterol*emia and large xanthomas round elbows and knee joints*, moreover, for the last 7 years he had been suffering from a progressive angina pectoris. At the autopsy macroscopically large lipoid and fibrous plaques were found all along the aortic *course, and distally increasing very much*. The pulmonary artery also showed vigorously lipoid alterations both proximally and in the peripheral ramifications but no fibrous plaques were observed here.

The patient with hypertension in the pulmonary circulation was a 24 year old woman who had had rheumatic fever when she was 9 years old and had been suffering from progressive heart trouble for the last 8 years. At the autopsy a vigorously stenosed and calcified mitralostium was found. The aorta was macroscopically normal with a well preserved elasticity, whereas the pulmonary artery showed numerous lipoid plaques especially peripherally.

The tissue specimens were all fixed in 4 per cent formaldehyde solution and some samples in 4 per cent basic lead acetate solution. All the tissue specimens were embedded in paraffin and cut in sections at 5 μ. The following stainings have been employed for the examination of ground substance, fibrils and cells:

Formalin fixed tissues:

Periodic acid Schiff's staining (PAS)
Alcian blue staining (72),
PAS combined with Alcian blue *staining* (6)
Hale's staining (33) modified by *Mowry* (53)
Eskelund's modification of Gomori's aldehyde fuchsin staining (18)
Silver *staining ad modum Foot*
van Gieson Hansen's staining
Hematoxylin eosin staining

Lead acetate fixed tissues:

Toluidine blue staining

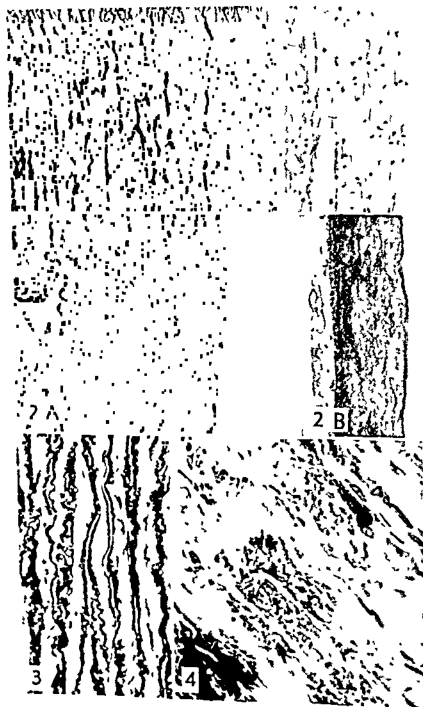
For staining of lipoid we used Sudan III and Alizarin red (73) (contrast coloured with toluidine blue) was used to demonstrate calcium.

A good control of the specificity of each individual method was obtained by employing the above mentioned staining methods, several of which generously supplement each other. It seems as if—in several cases—staining with Alcian blue does not give a perfectly exact picture of the total distribution of the acid mucopolysaccharides, whereas Hale's colloidal iron staining colours not only acid mucopolysaccharides but sometimes even some of the neutral glycoproteins. The author's experience is that by employing both these methods simultaneously with toluidine blue staining, the best possible impression of the distribution of hexosamine containing carbohydrates in vascular tissue is obtained.

To estimate collagen and reticular fibrils as well as component parts of the PAS positive substance, viz. glycoproteins, young collagen fibrils, reticular fibres and musculature, we used van Gieson Hansen, combined PAS Alcian blue and silver staining, one method supplementing the other.

In several cases calcium staining with Alizarin red was controlled partly by De ... by a preceding decalcification with fo ... on frozen sections with as well as without fixation in 4 per cent formaldehyde solution.

In several cases the sections fixed in basic lead acetate were *incubated* with hyaluronidases (bacterial hyaluronidase and/or testicular hyaluronidase) to determine the content of hyaluronic acid and chondroitin sulphuric acids of the ground substance. *Furthermore* some sections were *incubated with ribonucleases* (Ribonuclease Sigma 5 × crystallized Lot R 85 112) to exclude the ribonucleic acid part in the metachromasia. The enzymes were dissolved in McIlvaine's Standard Buffer (pH 6.5). The bacterial hyaluronidase was *prepared by Dr. J. Faber* (21) of the Danish State Serum Institute, Copenhagen. The testicular enzyme (Penetrase Leo Batch No. 590427 containing 500.000 i.u./g) was manufactured by Leo Pharmaceutical Products, Copenhagen.


2 A
2 B
3
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Figs 1–4

Fig 1 The thoracic aorta (48 years) (Eskelund's modification of Gomori's aldehyde-fuchsin staining × 140) The elastic fibres in the media are ruptured and fragmentated The internal elastic membrane is fragmentated and some elastic fragments are seen *profoundly in the intima* The cells both in the intima and media are mostly fibroblasts

Fig 2 The thoracic aorta (a) and the pulmonary artery (b) (70 years) (Eskelund's modification of Gomori's aldehyde fuchsin × 35) In the *aorta* the ground substance in the media separates the elastic fibres and these are irregular and show fragmentation The intima is vigorously proliferated The internal elastic membrane is splitted up.—In *the pulmonary artery* the ground substance is very scanty The internal elastic membrane is distinct and the elastic fibres are closely packed and show a regular course The intima is not proliferated but consists of a scanty subendothelial zone of mucoid substance The difference in the thickness of the two elastic vessels is very clear

Fig 3 The thoracic aorta the media (75 years) (Foot's silver method × 350) A distinct argyrophil zone or network of reticular fibrils is found in close relation to the elastic fibres

Fig 4 The thoracic aorta the media (Alizarin red × 560) Starting calcification in the metachromatic ground substance among the elastic fibres

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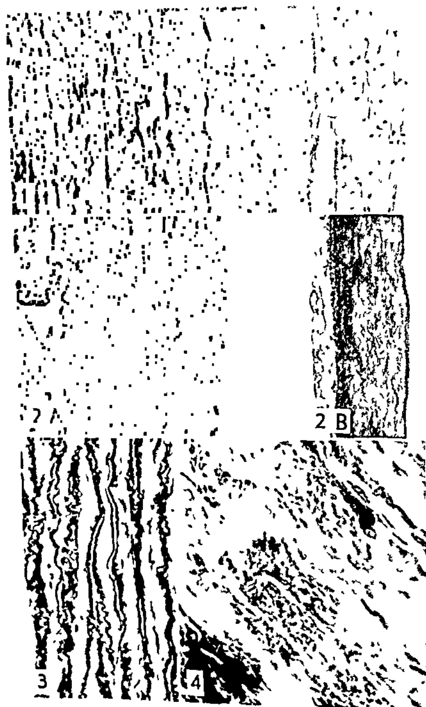
Even early in life calcium can be detected in the media In the vast majority *of cases an incipient mineralization can be demonstrated* about the age of 25 At the beginning the calcium appears as granules, deposited in the metachromatic ground substance, and with increasing depositing the calcium will occupy increasing amounts of the interfibrillar ground substance (Fig 4) *Even at rather pronounced medial calcification*, the course of the elastic fibrils is still seen to run *through* the deposits (Figs 5, 12) The mineralization seems to start rather diffusely in the intimal half of the media, spreading with age throughout the media

Comparisons between sections from abdominal and thoracic parts of the aorta will often show a difference of degree in the alterations, thus the accumulation of acid mucopolysaccharides, and more particularly the deposit of calcium and PAS-positive substance, is most pronounced in the abdominal section *This is especially conspicuous in the Alizarin-red staining of young aortae, where incipient calcium deposits are often observed in the abdominal part, whereas calcium cannot yet be detected in the thoracic part*

Intima

The stage of proliferation Immediately after birth a proliferation of the intima starts, increasing constantly with age This proliferation is of a rather uniform thickness throughout the vessel, and during the first two decades it consists of fibroblasts and metachromatic ground substance

The internal elastic membrane shows a rather vigorous rupture, and fragments of elastic fibrils are visible in the profound part of the intima (Fig 1) Moreover, an abundant amount of reticular fibres are


1
2 A
2 B
3
4

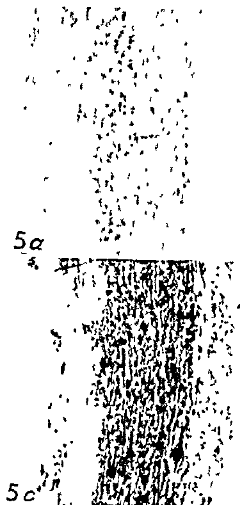




Fig 5
(× 35)
De
ce 3
59 year old person (cf Tables 1 and 2)

observable in young aortae, and with increasing proliferation, fragments of collagen fibrils make their appearance

The proliferating intima is soon subjected to a lipoid accumulation Starting in infancy, it grows steadily with increasing years (Fig 11) The lipoid may be intracellular in the so-called "foam cells", but with increasing amounts it appears in large, extracellular, homogenous lots, with a few "foam cells" peripherally located (Fig 6) The so-called "foam cells" are present as large cells with light cytoplasma and excentrically located nucleus With increasing lipoid accumulation in the intima a slight invasion of lipoid can—as mentioned—be observed in the luminal third of the media

Experimenting on animals, several authors have described a particularly marked accumulation of ground substance round the lipoid lots of the intima *In human aortae no reaction of connective tissue is visible in the intima round the accumulations of lipoid, neither an increase in the cellular elements, nor in the ground substance* (Fig 6)

The stage of fibrosis With increasing proliferation a gradual ac-

cumulation of PAS positive substance is observed to take place profoundly in the intima (Fig 9) This phenomenon becomes more pronounced with age, and fibroblasts with vigorous PAS positive cytoplasma and surrounded by PAS positive substance are often seen dispersed in the intima (Figs 7, 13) Fragments of PAS-positive fibrils appear in the intima with increasing content of PAS positive substance (Fig 14)

The thickness of the intima increases gradually during the stage of fibrosis, and the lipoid content is constantly increased both extracellularly and in lots of "foam cells" The tendency to a more profound depositing of lipoid in the intima is more widespread at this stage than at the proliferating stage At this stage—as in the proliferating stage—there is not found any signs of connective tissue reaction round the lipoid accumulations The intima round such an accumulation presents the normal distribution of ground substance, PAS positive substance, and fibrils

Neither in the macroscopically normal aortae, nor in macroscopically normal parts from atherosclerotic aortae is found the least trace of calcium deposits in the intima

The Relationship between Deposits in the Media and Intima.

In Table 1 an attempt has been made to give a schematic presentation of the relationship between the deposit of calcium in the media and the age of the individual in macroscopically normal parts of the thoracic aortae Furthermore we have stated the intimal changes, divided up into the stage of proliferation, in which the intimal thickening chiefly consists of acid mucopolysaccharides, and the stage of fibrosis, in which PAS positive substance appears and gradually dominates the picture According to a purely subjective estimate, the content of calcium in the media has been stated in the following degrees of intensity 0, 1, 2, and 3 (Fig 5)

The table shows that the calcium deposit makes its appearance in the media about the age of 20–30 [illegible]
that the intimal fibrosis only sta[illegible]
progressing Only when there is [illegible]
media the PAS positive fibrils in the intima can be observed

A distinct increase in the amount of PAS positive medial substance takes place simultaneously with the medial calcification

Table 2 further shows alterations in macroscopically normal parts from atherosclerotic aortae, and on comparing these with corresponding normal aortae no difference is observable in the age-alterations

A comparison between sections from the thoracic and the abdominal part will show a slight difference of degree in the alterations This applies to the deposit of calcium in the media as well as to the alterations in the intima

TABLE 1

The Relation in Various Age Groups Between the Intimal Alteration and the Calcification in the Media of Aortae with Normal Gross Appearance (5 cm below Arcus Aortae)

Age	Number	Degree of medial calcification (cf fig 5)	Number	Intima		
				Acid m p s	Glycoproteins	Fragments of PAS-pos fibrils
0–10	22	0	22	+		
		1				
		2				
		3				
11–20	10	0	9	+		
		1	1	+		
		2				
		3				
21–30	8	0	4	+		
		1	4	+	+	
		2				
		3				
31–40	7	0				
		1	1	+		
		2	6	+	+	
		3				
41–50	6	0				
		1	1	+	+	
		2	2	+	+	+
		3	3	+	+	+
51–60	7	0				
		1	1	+	+	
		2	1	+	+	+
		3	5	+	+	+
61–70	4	0				
		1				
		2				
		3	4	+	+	+
71–80	6	0				
		1				
		2				
		3	6	+	+	+
81–90	1	0				
		1				
		2				
		3	1	+	+	+

TABLE 2

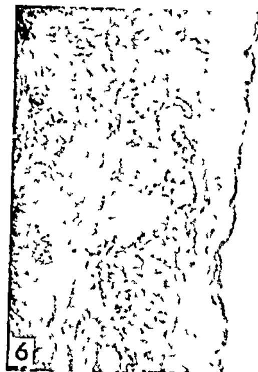
The Relation in Various Age Groups Between the Intimal Alteration and the Calcification in the Media of Parts with Normal Gross Appearance from Atherosclerotic Aortae (5 cm below Arcus Aortae)

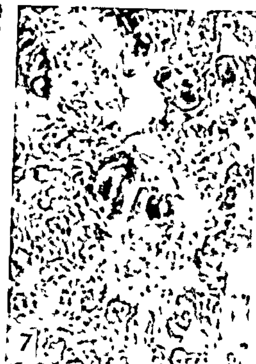
Age	Number	Degree of medial calcification (cf fig 5)	Number	Intima		
				Acid m p s	Glycoproteins	Fragments of PAS-pos fibrils
31 40	2	0				
		1	1	+	+	
		2	1	+	+	
		3				
41 50	7	0				
		1				
		2	1	+	+	
		3	6	+	+	+
51 60	4	0				
		1				
		2				
		3	4	+	+	+
61 70	13	0				
		1				
		2				
		3	13	+	+	+
71 80	10	0				
		1				
		2				
		3	10	+	+	+
81 90	7	0				
		1				
		2				
		3	7	+	+	+

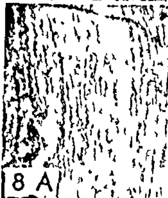
There cannot by comparing sections from macroscopically normal aortae and from macroscopically normal parts of atherosclerotic aortae be demonstrated any difference in the alterations with age The atherosclerotic aortic wall reveals a deposit normal to the age group of both calcium in the media and acid mucopolysaccharides and glycoproteins in the media and intima Furthermore, in normal parts of atherosclerotic aortae is observed an accumulation of lipoid in the intima accumulating in lots profoundly in the intima with increasing age but the amount of lipoid is not observed to be larger than in corresponding aortae with normal gross appearance

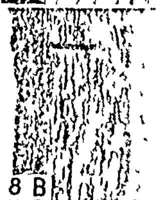
Pulmonary Artery

The age alterations in the pulmonary artery present many points of similarity with those found in the aorta The elastic fibrils particularly









Figs 6–8

Fig 6 The thoracic aorta the intima (24 years) (PAS Alcian blue × 140) Accumulation of lipoid in the deep part of the intima There is no reaction in the connective tissue The intima is in the stage of proliferation

Fig 7 The thoracic aorta the intima (PAS Alcian blue × 1100) Accumulation of PAS pos cells (Same picture as fig 13)

Fig 8 The pulmonary artery (The central (a) and peripheral part (b)) from the patient with hypertension in the pulmonary circulation (Eslelunds modification of Gomori's aldehyde fuchsin × 35) The ground substance separates the elastic fibres The inter nal elastic membrane is *fragmentated* and the elastic fibres are fragmentated and *ruptured* The *intima* is *vigorously* proliferated

the internal elastic membrane, remain far more regular with age, without the strong fragmentation and duplication *Even the pulmonary artery from elderly persons present only a slight intimal proliferation, and the accumulation of interfibrillar ground substance in the media is also rather scanty* (Fig 2)

Morphological structures and biochemical substances, similar to those in the aorta, are found The intima abounds in reticular fibres, whereas the amount of collagen fragments is very scanty In the interfibrillar ground substance in the media, reticular fibres, as well as sporadic collagen fragments, are observed, often in close relation to the elastic fibrils The glycoprotein deposit is scanty in the media, and not visible in the intima The ground substance contains both hyaluronic acid and chondroitin-sulphuric acids

Even in the pulmonary artery from very old persons only scanty accumulations of lipoid are observable in the intima, and in the present material no calcium is found in the media

The microscopical examination of vessels from the patient with Muller Harbitz's syndrome shows that in macroscopically normal parts from the aorta as well as from the pulmonary artery the entire intima and the innermost part of the media have plenty of lipoid, by far the largest amount to be found in the aorta The intima shows no proliferation except what is normal for the corresponding age group in the normal age material The media presents alterations ordinarily normal to the age

In the case with hypertension in the pulmonary circulation the aorta presents ordinary alterations normal to similar age groups Macroscopically normal tissue from the pulmonary artery, however, contains small amounts of lipoid throughout the intima, which is vigorously proliferated Moreover, large amounts of acid mucopolysaccharides are found in the media (Fig 8, cf Fig 2b)

Nature of the Histochemical Substances

The ground substance which is found partly to react metachromatically and Alcian blue-positively, and partly to be positive to Hale's colloidal iron, is composed of acid mucopolysaccharides, and incubation experiments with testicular and bacterial hyaluronidases show that there are both sulphate-free and sulphate-containing mucopolysaccharides, and that—in ageing—ratio Ch HA[1] greatly increases All these phenomenons are mentioned more explicitly in previous studies (6, 7)

Recent years' investigations (16 27) on human connective tissue have shown that it contains a number of neutral mucopolysaccharides, i e uronic acid free carbohydrates *Glegg et al* (27) have demonstrated

[1] Ch HA chondroitin sulphuric acids hyaluronic acid

that there is a distinct relationship between the content of neutral *mucopolysaccharides in a tissue*, and its ability to take stain by Schiff's reagent after oxidation with periodic acid

Bertelsen (5), in a previous study, isolated a strongly PAS-positive substance from the aorta Besides hexosamine, it contained different sorts of both keto- and aldohexoses, but no uronic acid

The increasing PAS-positive *substance in the media is likely to consist* chiefly of reticular fibres and neutral mucopolysaccharides Moreover, the smooth musculature reacts faintly PAS-positively

At first the PAS-positive substance in the intima is mainly composed of neutral mucopolysaccharides, but gradually the PAS-positive fibrils mentioned make their appearance In van Gieson-Hansen's staining they are suggestive of collagen fibrils, but owing to their fairly vigorous PAS-positive reaction, they must—unlike collagen substance—contain *neutral carbohydrates*

As mentioned above, frequently occurring cells are cells containing, or surrounded by, a PAS-positive substance So, the formation of the neutral mucopolysaccharides seems certainly to take place in the fibroblasts in the media and intima

The acid mucopolysaccharides are also certain to be created locally by the fibroblasts In young aortae sporadic metachromasia is often visible round the fibroblasts, whereas intracellular metachromasia has *never been observed*

The Relationship between Macroscopical and Microscopical Changes of the Aorta

The aorta from a young individual is fairly thin and very elastic In ageing the actual wall thickness increases and the elasticity decreases proportionally The media which must *be considered the actual supporting tissue of the aorta* is the decisive factor in this alteration Consequently the deposit of calcium in the media must greatly influence the elasticity and this presumption is also confirmed by *Wilens* (74) investigations He described a decrease with age in the elasticity of human aortic tissue, and this seems to be in complete accordance with the increasing calcium content in the media

Besides the decreasing elasticity in the aorta a distinct change of the intima is observed with increasing age In young aortae it is very difficult to separate the intima from the media but with increasing years it becomes easier to remove the intima as a compact fibrous fairly strong membrane

DISCUSSION

Bertelsen & Jensen (6, 7) *in previous publications*, have demonstrated that the thickening of both the intima and media largely consists of sulphate-free as well as sulphate containing mucopolysaccharides They further found that the ratio Ch/HA increases considerably with age, it should, however, *be pointed out* here, that even at a very advanced age and at vigorous sclerosis, hyaluronic acid can always be detected in aortic tissue *Bertelsen* (5) demonstrated in a later study that the aortic content of both uronic-acid-free *and uronic acid containing carbohydrates* is increasing with age

It is universally acknowledged that mucopolysaccharides tend to accumulate in tissue with a low oxygen tension, and that this particularly applies to sulphate-containing mucopolysaccharides (1). The distribution of vasa vasorum in the aortic wall (25, 63, 76) seems to indicate that the oxygen tension is low in the intima and the luminal half of the media, which may possibly account for the large accumulation of mucopolysaccharides in these strata. The increased content of these substances will certainly further hamper the blood supply to the vascular wall.

This supposition is supported by the fact that the content of mucopolysaccharides is largest in the intima and the luminal half of the media, whereas the medial part facing the adventitia does not present major deposits. The adventitia itself contains no, or only a scanty amount of mucopolysaccharides. Several experimental works (4, 48, 51, 64), too, support the supposition that anoxia in the vascular wall may be of importance to the accumulation of acid mucopolysaccharides in the media.

The intimal proliferation and the later fibrosis, which especially increases after the medial calcification has started, may thus easily be explained as a consequence of the ischemic condition which prevails in the vascular wall, and which is further aggravated by a depositing of calcium in the media.

The influence of the pressure on the vascular content of mucopolysaccharides, cf. the aorta versus the pulmonary artery, may possibly be explained by the fact that an increased pressure in a vessel will decrease the drenching of the vascular wall.

Whether the decreased oxygen tension in tissue is the immediate cause of an increase in the production of mucopolysaccharides or whether this is due to an indirect accumulation of various residual products, which secondarily stimulates the fibroblasts to formation of mucopolysaccharides, is a question that will not be considered here. The author only wants to point out that a decreased oxygenic tension in tissue will result in increased glycolysis with formation of lactic acid, which may potentiate the formation of mucopolysaccharides (1).

Since the publication of *Virchow's* (69, 70) studies the question of the pathogenesis of the lipoid accumulation, and its relation to the intimal proliferation has been lively discussed.

Virchow and several of his contemporaries (14, 38, 39, 40) spoke about a "lipoid degeneration" which secondarily stimulated to formation of connective tissue. The calcium was then deposited close to the lipoid.

Faber (19), and later *Ribbert* (55), presumed that the accumulation of lipoid and the formation of connective tissue were independent of each other. *Schultz* (65), *Ssolowjew* (67) and *Wolkoff* (77), held that the mucoid substance, increasing with age in the vascular wall, had "affinity" to lipoid.

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place in the human organism on the basis of carbohydrates (*Rud* (6?)) Both the endothelial cells and the fibroblasts in the intima may function as such an active cellular element

The increased lipoid deposit present in the intima at lipoidosis (Muller Harbitz s syndrome), speaks greatly in favour of the presumption that the lipoid is present as a filtration product whereas the increased lipoid deposit at an increase of blood pressure may just as well be due to a local formation

A comparison between on one hand the vigorous accumulation of acid mucopolysaccharides in both the intima and media in the aorta and on the other hand the very moderate accumulation in the pulmonary artery will distinctly reveal the great quantitative differences Both vessels contain the same cellular and fibrillar elements and the distribution of vasa vasorum is largely the same As will be known the blood pressure to which the pulmonary artery is exposed is appreciably lower than the pressure in the aorta and it is an idea which immediately suggests itself that the difference in the histological picture are resulting from the difference of the pressures in the two vessels Whether the influence of the pressure on the formation of mucopolysaccharides—as already mentioned—is due to an increased anoxia in the vascular wall or to a stimulation of the cellular elements of the vascular wall cannot be decided

Experimental atherosclerosis (2 10 11 15 23 31 36 75) made principally on rabbits gives fairly concurrent results *viz* both hypercholesterolemia and hypertension produce a tendency to lipoid deposit in the intima and the two factors mentioned seem to accelerate each other Present findings seem to indicate that—to a certain degree this also applies to human vessels as lipoidosis causes a tendency to considerable lipoid deposits in the intima most abundantly where the blood pressure is highest At normal plasmalipoid lipoidosis is also observed in the aorta where the blood pressure is considerably higher than in the pulmonary artery where even very advanced age does not cause a deposit of lipoid in the intima at normal blood pressure in the pulmonary circulation

Lansing et al (41 42 43 44 45) are of the opinion that the calcium in the aorta is localized to the very elastic fibril To prove this they use partly microincineration and partly biochemical methods They isolate and produce in pure form elastin by basic hydrolysis The fact that this elastin contains large quantities of calcium—increasing strongly with age is not as the authors hold due to a deposit of calcium in the elastic fibrils but to the fact that calcium is insoluble in basic surroundings The calcium deposited in the ground substance between the elastic fibrils will thus by alkaline hydrolysis be precipitated together with the elastin and it is therefore present as an impurity and not as a component of the fibrils

Present findings show that the calcium is deposited in the ground

Numerous animal experiments, in which atherosclerosis in the aorta was produced by feeding with cholesterol and/or increased blood pressure, *have been made during recent decades*. *Buck* (11) found that *both lipoid and metachromatic* substance were present in macrophages, which—on attaining a certain size—liberated both the lipoid and the *metachromatic* substance to the surroundings, where the lipoid should further stimulate the fibroblasts to formation of mucopolysaccharides. *Wilens* (75) presumed that the intimal thickening is an integrating part of *the* artherosclerosis, preceding the deposit of lipoid. *Gore* (31) held that mucopolysaccharides were secondary in relation to the lipoid deposition, which primarily appeared in big macrophages, directly below the endothelium.

In their publications, however, *Rinehart & Greenberg* (57), *Fisher et al.* (23), and *Moon & Rinehart* (52) concluded that a primary accumulation of mucopolysaccharides was later followed by an occurrence of increased content of lipoid in the ground substance.

The present investigations seem to confirm that formation of connective tissue and accumulation of lipoid are independent of each other, though they are often observed simultaneously in the vascular wall. Partly *does the intimal proliferation commence before lipoid* can be demonstrated in the vessel, and partly is the intimal proliferation uniformly distributed on the total vascular surface, whereas the accu*mulation of lipoid is often concentrated in lots, round* which no tendency to deposits of mucopolysaccharides are observed.

In the above case of Muller-Harbitz's lipoidosis a vigorous accumulation of lipoid was *observed in the aortic intima*, whereas *the accumulation* of mucopolysaccharides was no larger than the proliferation in normal aortic from similar age-groups. As mentioned there was also large amounts of lipoid in *the* pulmonary artery, *but almost no intimal* proliferation.

Whether the lipoid is transported via the passing plasma to the intima and deposited there, or whether it is a locally cellular product, cannot be decided from the present investigation. By far the majority of authors support the theory that lipoid filters into the intima from plasma and is accumulated there. In 1948, *Faber* (20) advanced the theory that heparin in the vascular tissue forms a protein-complex with lipoproteins, by which the lipoid is liberated. He based his theory on experiments made by *Chargaff* (12, 13). Some local factors must certainly be supervening at the depositing of the lipoid, for in many other parts of the organism we find accumulations of mucopolysaccharides without any tendency to lipoid accumulation. It is conceivable that the endothelial cells are here functioning as a possible active connecting link, carrying the lipoid into the intima.

It is very likely that the depositing of lipoid is caused by a local cellular reaction, for investigations—made during recent years into the lipogenesis—have established that an extrahepatic lipogenesis takes

fibres, this can be observed in silver-staining as well as in staining with Schiff's reagent after oxidation with periodic acid *Gillman et al* (26), experimenting on rats, made a similar finding

As will be known, collagen fibrils are composed of protein substances without content of carbohydrates (16) Quantitatively hydroxyproline, proline, and glycin are the most abundantly represented aminoacids The faint PAS positive reaction of collagen fibrils, seen particularly in fairly young individuals, is evidently caused by a content of amino acids with 1, 2 glycol groups in the collagen protein, or the collagen molecules are combined with carbohydrate material, which by periodic acid are oxidized to dialdehyde and/or diketonic groups (37, 49)

Even though the acid mucopolysaccharides do not form part of the collagen fibrils they are universally presumed to be of essential importance to the formation of fibrils (32 35, 50) It is anticipated that the carbohydrate-macromolecules act as a "pattern" according to which the collagen-protein molecules are orientated or the fibroblasts deposited *Meyer* (50) holds that chondroitin sulphate takes part in the fibrillogenesis, and he finds also that young, unripe, easily soluble collagen is chiefly associated with chondroitin sulphate A and C, whereas older, coarse fibrils most frequently are connected with chondroitin sulphate B

As mentioned, the mineralization is dependent on several factors, of which only a few are known When it starts in the media, all factors known to be essential to the crystallization are present in the tissue Ratio Ch.H.A has been shifted to the advantage of the chondroitin-sulphuric acids, and the ground substance contains abundant amounts of collagen fibrils The inorganic factors are presumably present in more than abundant quantities, so that the crystallization starts at a time that evidently depends on a mutual interaction between "local factor(s)" Above mentioned investigations seem to show that the optimal conditions for an incipient mineralization is present in the course of third decade

Whether the calcification starts as a crystallization in or along the collagen fibrils, cannot with certainty be decided by ordinary histological methods, submicroscopical methods must therefore be employed for this purpose The result of such investigations will appear from forthcoming publications

A comparison between the changes in the aorta and the pulmonary artery will reveal many similarities Both vessels present a rupture of the elastic fibrils and an accumulation of acid mucopolysaccharides in the media as well as in the intima — but the changes are by far the greatest in the aorta By increased pressure in the pulmonary circulation an increasing amount of acid mucopolysaccharides are observed in the pulmonary artery, it must therefore be assumed that the larger content of acid mucopolysaccharides in the aorta is partly attributable to the high pressure which prevails in the aorta

substance between the elastic fibrils The fact that an increasing mineralization may result in a picture which suggests a deposit of calcium outside the elastic fibrils, is exclusively a quantitative phenomen

The calcification of the media is a typical mineralization, by which is understood a depositing of inorganic crystals in or on an organic matrix Newer investigations (28, 29, 61) into normal calcium deposits in certain organic tissues (bone, dentine, and enamel) show that the process takes place in an organic matrix, consisting partly of ground substance, and partly of collagen fibrils The ground substance contains large quantities of sulphated mucopolysaccharides Electronmicroscopical examinations (58, 60) show that the mineralization starts on the surface of the collagen fibrils, all the crystals being orientated with their axes placed longitudinally along the fibrils Later investigations (59) seem to indicate that the crystallization of calciumsalts may start inside the collagen fibril, and that it is not localized only to the surface

How important a role the ground substance, *i e* the chondroitin sulphate in which the collagen fibrils are imbedded, plays for the mineralization-process is still to a certain degree an open question

Several experiments (9, 29, 68) seem to indicate that besides collagen fibrils, chondroitin sulphate and a sufficiently high concentration of inorganic materials (Ca^{++}, PO_4 , CO_3), a still unknown *"local factor('s)" is necessary for the start of the mineralization Finbinder et al* (17) have demonstrated that protein-collagen and chondroitin sulphate have great affinity to each other and form complex compounds, and *Sobel* (66) in *in vitro* experiments shows, besides what has just been mentioned, that the collagen-chondroitin sulphate complex may be part of "local factor" It is conceivable that the pressure in the aorta may be such a "local factor"

Chondroitin sulphate forms—in combination with tissue protein—a gel with a conspicuously large amount of free aniongroups *In vitro* experiments (9) with hyaline cartilage, containing large amounts of chondroitin sulphate, show that it may bind large quantities of calcium-ions from a calcium solution The presumption that vascular chondroitin sulphate binds large amounts of calcium-ions which are liberated again for some cause, first by depolymerization of chondroitin sulphate, is supported by the *in vitro* experiments made by *Glimcher* (28 29), these experiments show that it is possible to produce calcification of highly collagen tissue by depolymerization of the ground substance

As mentioned, increasing amounts of collagen fibrils in the aortic ground substance are found with increasing age *Hass* (34) thinks that the collagen fibrils are located chiefly along the elastic fibres This is partly incompatible with present observations, which show that the collagen fibrils are uniformly distributed in the ground substance among the elastic fibres The author and his collaborator (24) find that the elastic fibres are surrounded by a compact network of reticular

fibres this can be observed in silver staining as well as in staining with Schiff's reagent after oxidation with periodic acid *Gillman et al* (26), experimenting on rats, made a similar finding

As will be known, collagen fibrils are composed of protein substances without content of carbohydrates (16) Quantitatively hydroxyproline, proline, and glycin are the most abundantly represented aminoacids The faint PAS positive reaction of collagen fibrils, seen particularly in fairly young individuals, is evidently caused by a content of amino acids with 1, 2 glycol groups in the collagen-protein, or the collagen molecules are combined with carbohydrate material, which by periodic acid are oxidized to dialdehyde and/or diketonic groups (37, 49)

Even though the acid mucopolysaccharides do not form part of the collagen fibrils, they are universally presumed to be of essential importance to the formation of fibrils (32, 35, 50) It is anticipated that the carbohydrate macromolecules act as a "pattern" according to which the collagen protein molecules are orientated or the fibroblasts deposited *Meyer* (50) holds that chondroitin sulphate takes part in the fibrillogenesis and he finds also that young, unripe, easily soluble collagen is chiefly associated with chondroitin sulphate A and C, whereas older, coarse fibrils most frequently are connected with chondroitin sulphate B

As mentioned the mineralization is dependent on several factors, of which only a few are known When it starts in the media, all factors known to be essential to the crystallization are present in the tissue Ratio Ch/HA has been shifted to the advantage of the chondroitin sulphuric acids and the ground substance contains abundant amounts of collagen fibrils The inorganic factors are presumably present in more than abundant quantities so that the crystallization starts at a time that evidently depends on a mutual interaction between "local factor('s) Above mentioned investigations seem to show that the optimal conditions for an incipient mineralization is present in the course of third decade

Whether the calcification starts as a crystallization in or along the collagen fibrils cannot with certainty be decided by ordinary histological methods submicroscopical methods must therefore be employed for this purpose The result of such investigations will appear from forthcoming publications

A comparison between the changes in the aorta and the pulmonary artery will reveal many similarities Both vessels present a rupture of the elastic fibrils and an accumulation of acid mucopolysaccharides in the media as well as in the intima,—but the changes are by far the greatest in the aorta By increased pressure in the pulmonary circulation an increasing amount of acid mucopolysaccharides are observed in the pulmonary artery, it must therefore be assumed that the larger content of acid mucopolysaccharides in the aorta is partly attributable to the high pressure which prevails in the aorta

It seems quite likely that the high pressure in the aorta is essential to the mineralization in the media as "local factor", but whether the ensuing intimal fibrosis is caused by the calcareous deposit in the media, perhaps combined with the pressure, cannot be decided from the present material

It will appear from the above investigations that it is difficult collectively to elucidate the age-alterations in the elastic vascular tissue, since changes, caused by the pressure in the vessel, dominate the histological picture We may leave out of account the different physiological conditions acting on the aorta and the pulmonary artery, and define the age-alterations of each single vessel separately, as alterations, seen in macroscopically normal tissue Thus defined, the pressure alterations are considered part of the age-alterations

CONCLUSION AND SUMMARY

The author attempts to elucidate the age-alterations in macroscopically normal tissue from the aorta and the pulmonary artery According to the above results it seems likely that the lipoid accumulation in the intima of the aorta and the changes in the connective tissue of the media-intima are two independent processes taking place simultaneously in the vascular wall The accumulation of lipoid is observable diffusely in the intima of the aorta at a very early age, increasing uniformly in quantity, and at the same time showing a tendency to accumulate in small lots profoundly in the intima

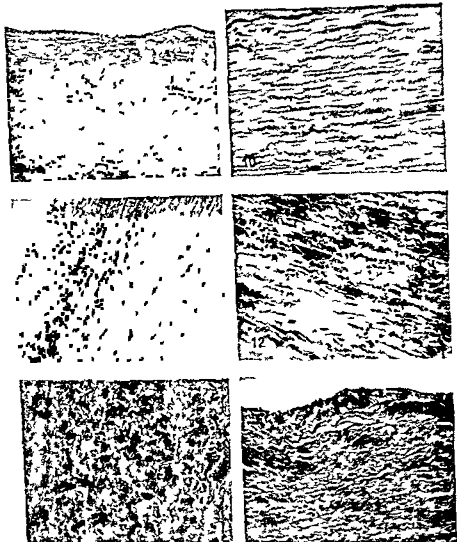
The lipoid accumulation is discussed, it may be caused either by a filtering or by a locally cellular formation

Connective tissue reaction has not been demonstrated round the lipoid deposits, neither in the stage of proliferation, nor in the stage of fibrosis

Whether the connective tissue alterations, consisting primarily of an accumulation of acid mucopolysaccharides, secondarily of glycoproteins, may be a basis-lesion for a later formation of intimal plaques, cannot be decided from the present material The accumulation of acid mucopolysaccharides is discussed against the low oxygen-tension prevailing in the vascular wall

Calcium is found very early in the media of the aorta, and a theory is advanced to the effect that the deposit of calcium here is a normal mineralization-process in an organic matrix, and that the calcium crystals are deposited in the chondroitin sulphate in close relation to the collagen fibrils The crystallization of calciumsalts is dependent on collagen fibrils and chondroitin sulphate, as well as on the concentration of inorganic material and one or several "local factor('s)", still unknown to us

The intimal alteration, starting at a very early age, is greatly accelerated by the medial calcification It is discussed whether there is a



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F g 9 The thorac c aorta (32 years) (PAS Alc an blue × 40) W despread Alcian blue and PAS pos substance in the prol ferated nt ma A good deal of PAS pos spots are seen deeply in the nt ma Alc an blue po and PAS pos substance among h

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CONCLUSION AND SUMMARY

The author attempts to elucidate the age alterations in microscopically normal tissue from the aorta and the pulmonary artery. According to the above results it seems likely that the *lipoid accumulation in the intima of the aorta and the changes* in the connective tissue of the *media-intima* are two independent processes taking place simultaneously in the vascular wall. The accumulation of lipoid is observable diffusely in the intima of the aorta at a very early age increasing uniformly in quantity and at the same time showing a tendency to accumulate in small lots profoundly in the intima.

The lipoid accumulation is discussed; it may be caused either by a filtering or by a locally cellular formation.

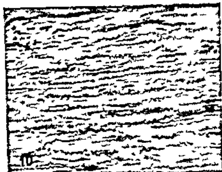
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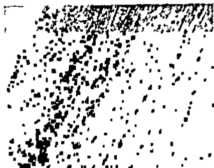
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Calcium is found very early in the media of the aorta and a theory is advanced to the effect that the deposit of calcium here is a normal mineralization process in an organic matrix and that the calcium crystals are *deposited* in the chondroitin sulphate in close relation to the collagen fibrils. The crystallization of calcium salts is dependent on collagen fibrils and chondroitin sulphate as well as on the *concentration of inorganic* material and one or several local factor(s) still unknown to us.

The intimal alteration starting at a very early age is greatly accelerated by the medial calcification. It is discussed whether there is a













Figs 9 14

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direct relationship between the medial calcification and the changes in the intima, or whether they are but the result of a common cause

It seems to be a fairly theoretical division to distinguish between ordinary age-alterations in the aortic wall, and alterations, specifically connected with the sclerotic process, since there are many indications that the *fibrous plaque is the final result of a process*, commencing at a very early age

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HISTOCHEMICAL INVESTIGATIONS ON PLAQUES IN HUMAN ATHEROSCLEROTIC AORTAE

By

SV BERTELSEN

Received 2 viii 60

In a previous histochemical study *Bertelsen* (3) demonstrated the medial calcification in aortic tissue as a commonly occurring phenomenon in the course of the 3rd decade It was demonstrated that the intimal proliferation, which—until the deposit of calcium salts starts in the media—consists of fibroblasts and acid mucopolysaccharides, is considerably accelerated by the medial mineralization A depositing of glycoproteins starting profoundly in the intima takes place simultaneously with the increasing intimal proliferation

In the above publication were solely described changes in normal aortae or in macroscopically normal parts from atherosclerotic aortae In the present study it is attempted to elucidate changes, connected to the fibrous and lipoid plaques in the aorta comparing them with the previous findings

MATERIALS AND METHODS

The material was obtained fresh at autopsy at the Rigshospital and the Copenhagen [illegible]

plaques several of the samples were taken as serial specimens at different distance from the plaques besides through the actuel plaques Some of the specimens wer decalcinated in formic acid and sodium formiate before the fixing

Age Distribution of the Material

Age	Number
41 50	9
51–60	7
61 70	16
71 80	10
81 90	7

It was the hope that partly by making serial specimens and serial sections an partly by using decalcification prior to fixation it would be possible to throw ligh on basal lesions connected to the atherosclerotic alteration

Aided by grant from the Danish State Science Foundation

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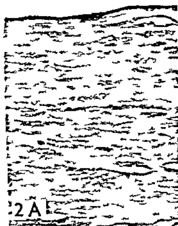
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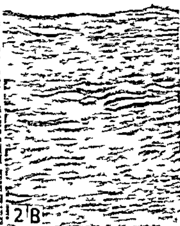
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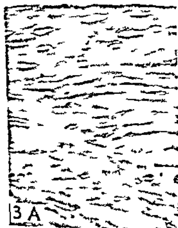
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1


2 A


2 B


3 A


3 B

In order to supplement the material both fibrous and lipoid plaques from aorta of a patient with Muller Harbitz's syndrome were examined The cases is further described in a previous publication (3)

The tissue specimens were fixed in 4 per cent formaldehyde solution and some of the samples in 4 per cent basic lead acetate The tissue specimens were imbedded in paraffin, cut in sections at 5 μ and stained with periodic acid Schiff (PAS) Alcian blue combined PAS Alcian blue, and Hale's staining modified by Mowry moreover with hematoxylin eosin van Gieson Hansen Ekelund's modification of Gomori's aldehyde-fuchsin and toluidine-blue Lipoid was demonstrated with Sudan III, and calcium with Alizarin-red (For further descriptions see previous publications (3))

Macroscopical Changes in the Aortae Examined

An atherosclerotic aorta may present different characteristical lesions, which the author attempts to arrange into two main groups, *viz*

(1) lipoid plaques (lipoidosis),
(2) *fibrous plaques* (sclerosis)

In connection with both forms of plaques, calcium, and perhaps also *atheromatous ulcers, may occur in the intima*

The lipoid plaque is observed as a yellowish, often slightly convex, cushion-shaped, soft thickening It seems frequently to be composed of numerous confluent plaquelets of a fairly moderate size The lipoid plaques may appear throughout the aorta, and when they have grown to a certain size, their tint may change from yellow to white The consistency then becomes distinctly more compact, and in connection with this, tangible calcium may occur, and perhaps *formation of* atheromatous ulcer

The fibrous plaque is whitish or greyish-yellow, and it is observed as an appreciably thickened, firm part of the aortic wall The size of the plaques may vary considerably, and the distribution of the plaques is found to be largest distally A yellowish, soft part may be formed centrally in the fibrous plaque Calcium and or perhaps formation of atheromatous ulcer may *also occur in connection with this plaque*

It appears *distinctly from the description that there is a great similarity between* the two forms of alterations, particularly fairly old lipoid plaques with fibrosis cannot macroscopically be distinguished from fibrous plaques The lipoid plaque may, in contrast to the fibrous plaque, appear at an early age particularly minor lipoid accumulations are often seen at an age of 20–30 as yellowish streaks in the intima

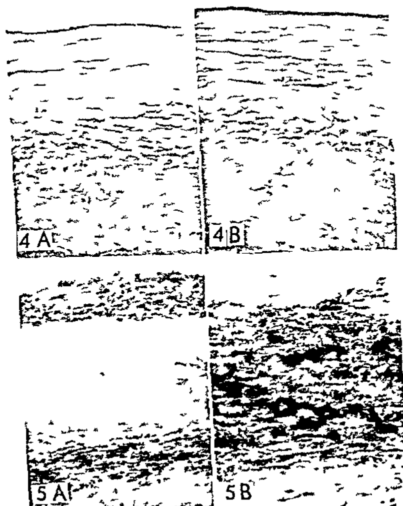
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Figs 1 3

Fig 1 Fibrous plaque (Ekelund's modification of Gomori's aldehyde fuchsin staining × 35) The border between the plaque and macroscopically normal aortic tissue is distinct

Fig 2 Fibrous plaque (*a* van Gieson Hansen × 140 *b* PAS Alcian blue × 140) Bunches of collagen like PAS pos fibrils in the intima in its entire *thickness*

Fig 3 Fibrous plaque (*a* van Gieson Hansen × 140 *b* PAS Alcian blue × 140) The intimal fibrils are increased in thickness and the central part of the fibres does not take either van Gieson *Hansen's stain or Schiff's reagent after oxydation* with periodic acid



Figs 4 5

Fig 4 Fibrous plaque (a van Gieson Hansen × 140 b PAS Alcian blue × 140) The normal fibrillar structure is obliterated and an atheroma is formed

Fig 5 Fibrous plaque with calcification (a Alizarin red × 35 b Alizarin red × 140 The thickening of the intima is pronounced The calcium salts are deposited in the superficial part of the intima in the scanty ground substance and in close relation to the fibres In picture a the deposit of calcium in the media is visible

as small deposits If an atheroma reaches as far down as the internal elastic membrane calcium will often be seen to "invade the atheroma profoundly from the media The formation of atheromatous ulcer is caused by an atheroma expanding towards the surface at last breaking through the endothelial stratum This will involve a draining of the atheromatous material and calcium—if any—feels like granulets or

RESULTS

Sections cut through *macroscopically normal* tissue present alterations mentioned in a previous publication (3), and only a short summary will be given below.

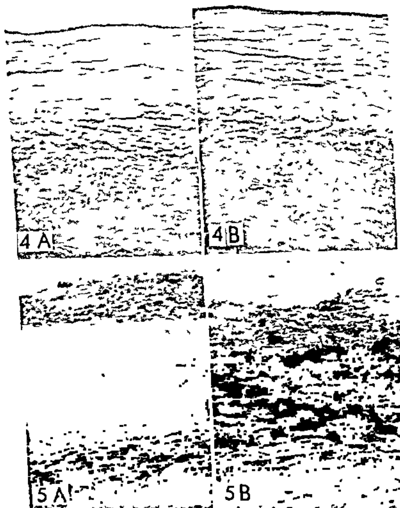
In the media is observed both an accumulation of acid mucopolysaccharides and glycoproteins, splitting the elastic fibrils from each other. The elastic fibrils are ruptured, and the ground substance abounds in collagen fibrils. Throughout the ground substance there are abundant amounts of fibroblasts and a few smooth muscle cells. Throughout the entire media a vigorous calcification is seen in the ground substance.

In the intima are found acid mucopolysaccharides and glycoproteins as well as fragments of PAS-positive fibrils, dependent on the age.

In connection with a *fibrous plaque* the thickening of the intima is markedly increased and serial sections at decreasing distances from the plaque, show a distinct increase in the PAS-positive fibrils and a decrease in the interfibrillar substance (Fig. 1). Through the actual plaque the course of the PAS-positive fibrils is seen to be very closely located through the whole intima, with only a slight amount of Alcian blue-positive and metachromatic substance among the fibrils (Figs. 8, 9). The actual fibrils are very vigorous, they appear rather swollen, and their course is running in regular, parallel streaks (Fig. 2). Only few cells are observable interfibrillarly. In van Gieson-Hansen's staining the fibrils are coloured like collagen. The fibrous plaque does not contain any elastic tissue. By staining with Sudan III the content of lipoid does not appear larger through the fibrous plaque than beside it.

Fibrils placed centrally in such a plaque are often seen to increase in thickness, while the central part of the fibres does not take either van Gieson-Hansen's stain or Schiff's reagent after oxidation with periodic acid (Fig. 3). This uncoloured part of the fibril consists of a homogeneous substance, and where this become very pronounced the normal fibrillar structure may be obliterated, and a large accumulation of an uncoloured, homogeneous tissue—an atheroma—is formed (Figs. 4, 10). In the present material it has not been possible to demonstrate any relationship between the content of lipoid in the intima and the mentioned connective tissue reaction with formation of atheromas. An atheroma will certainly contain larger or smaller amounts of lipoid dependent on the quantities of lipoid which the intima contained before the atheromatous formation.

Calcification in the intima is a rare phenomenon in proportion to the frequency of calcium deposits in the media. The mineralization starts round the PAS positive fibrils in the ground substance and appears but sporadically (Figs. 5, 11). An increasing deposit of calcium will obliterate the fibrillar structure, and at a formation of an atheroma calcium lots may "invade" this and be located sporadically



Figs 4 5

Fig 4 Fibrous plaque (*a* van Gieson Hansen × 140 *b* PAS Alcian blue × 140) The normal fibrillar structure is obliterated and an atheroma is formed

Fig 5 Fibrous plaque with calcification (*a* Alizarin red × 35, *b* Alizarin red × 140) The thickening of the intima is pronounced The calcium salts are deposited in the superficial part of the intima in the scanty ground substance and in close relation to the fibres In picture *a* the deposit of calcium in the media is visible

as small deposits If an atheroma reaches as far down as the internal elastic membrane, calcium will often be seen to "invade" the atheroma profoundly from the media The formation of atheromatous ulcer is caused by an atheroma expanding towards the surface, at last breaking through the endothelial stratum This will involve a draining of the atheromatous material, and calcium—if any—feels like granulets or





Figs 6–7

Fig 6 Lipoid plaque from the patient with Muller-Harbitz's syndrome (Lskelund's modification of Gomori's aldehyde fuchsin staining × 35) A large extracellular accumulation of lipoid with sclerosis of the superficial intima The fibres in the intima above the lipoid accumulation are distinctly PAS-pos and are coloured like collagen (cf fig 12) Profoundly in the lipoid accumulation is a precipitation of cholesterol crystals

Fig 7 Lipoid plaque from the patient with Muller Harbitz s syndrome (PAS-Alcian blue × 140) Accumulation of "xanthoma cells in the intima No reaction in the superficial part of the intima is visible

grains In the bottom of an atheromatous ulcer, the medial calcium may possibly be palpable

An atherosclerotic aorta will always present a vigorous mineralization of the media in its entire thickness, and no alteration in the calcareous intensity is observable, either below a plaque or beside it (Fig 5) This may be due to the fact that the medial calcium in our material is so widely dispersed that a further quantitative determination on histological sections is impossible Below a plaque the accumulation of PAS positive substance seems to be larger than in the other parts of the media, while the amount of acid mucopolysaccharides is decreased No alteration in the elastic fibrils has been observed They show the usual irregular course and rupture

A previous publication (3) mentioned the diffuse accumulation of lipoid in the intima starting already at an early age, and increasing constantly with age With increasing amounts of lipoid in the intima, it accumulates in lots, often profoundly located towards the internal elastic membrane These lots consist mainly of extracellular lipoid deposits with a few "foam cells" peripherally Gradually as the lipoid accumulation increases in size, that particular part of the intima is thickened, and a yellowish lipoid plaque is now macroscopically visible at the luminal side of the vessel This intimal thickening is not caused by a connective tissue reaction, but exclusively by the large amount of lipoid

Only at very large extracellular accumulations of lipoid and a simultaneous vigorous mineralization in the media, is a reaction observable in the superficially covering intima Bunches of collagen like, PAS-positive fibrils exactly correspond to the fibrillar accumulation at the fibrous plaque, are now distinctly visible (Figs 6 12) The fibrils often appear swollen, and atheromatous parts are frequently found Only scanty interfibrillar, Alcian blue positive ground substance is seen, and only few fibroblast like cells There are no elastic fibrils

The large lipoid accumulations with superficial intimal sclerosis will often—in contrast to the small accumulations without intimal reaction—show precipitations of cholesterol crystals (Fig 6)

Also in the secondarily sclerosed intima—above a lipoid accumulation—deposits of calcium crystals are observable in close relation to the fibrils a course which looks completely like the process in the fibrous plaque (Fig 13) Also here it is possible to observe a "invasion" of calcium into the lipoid accumulation, partly from the intimal calcium, and partly from the medial calcium (Fig 13)

At serial sections made at different distances from a lipoid plaque, with as well as without superficial sclerosis, the changes in the intima are seen to correspond completely with those of a similar age group from non-atherosclerotic vessels

The media shows alterations below the lipoid plaques as compared to corresponding age groups from non-atherosclerotic vessels Below













Figs 8 13

Fig 8 Fibrous plaque (PAS Alcian blue × 100) Distinct collagen like PAS pos fibrils in the intima The ground substance is Alcian pos

Fig 9 As fig 8 (Hale s staining modified by Mowry × 100) The scanty ground substances consists of acid mucopolysaccharides

Fig 10 Fibrous plaque (PAS Alcian blue × 40) The PAS pos fibrillar structure is indistinct and an atheroma is formed

Fig 11 As fig 8 (Alizarin red × 100) The mineralization starts with deposit of calcium salts in the ground substance in close relation to the fibrils

Fig 12 Lipoid plaque (Van Gieson Hansen × 40) Lipoid accumulation with sclerosis of the superficial intima The collagen like fibres are swollen and the central part of the fibrils does not take stain

- 1 × 40) Deposits of calcium salts crystals in relation intima A invasion of calcium into the lipoid of calcium in the media is seen

large fibrous and lipoid plaques the media may be tapered, so that it looks as if the plaque has expanded into the media This is presumably caused by atrophy of the media below the plaques

In the above mentioned case of Muller Harbitz's syndrome, all forms of lipoid accumulations were found in the intima, from accumulations of 'foam cells (Fig 7) and small extracellular accumulations without intimal reaction to large deposits of lipoid with cholesterol crystals and superficial, intimal fibrosis Moreover, several primary fibrous plaques were observed

DISCUSSION

From the above it will appear that in macroscopically normal tissue outside the plaques, the vascular wall is unaltered except for changes normal to the age This is in perfect accordance with the findings in a previous study (3)

The different fibrous alterations in the intima have quite different pathogenesis in spite of the above mentioned similarities in the histological picture

No connection has been detected between the lipoid content of the intima and the degree of atherosclerosis A lipoid laden intima is often observed without any universal signs of atherosclerosis conversely, a vigorous atherosclerosis may occur without any large amount of lipoid in the intima

Ophuls (11), in histological studies found a similar phenomenon He, too distinguished between the lipoid plaque and the fibrous plaque He is of the opinion that the deposit of lipoid is caused by a semi physiological process in the intima, resulting from disturbances in the cholesterol metabolism, whereas the fibrous plaque is characterized by a regeneration of fibrous tissue and perhaps necrosis Often the necrotic tissue contains ample amounts of lipoid

Anitschkow (1), in histological studies found many points of similarity between experimental cholesterol induced atherosclerosis in rabbits and atherosclerosis in human aortae Talking about experimental atherosclerosis he described the so-called 'xanthoma cells", which are often located peripherally of the extracellular lipoid accumulation in the intima Gradually an elasto fibrous stratum is formed superficially of the lipoid

By following the changes during 2–3 years after the cholesterol-feeding Anitschkow found that the intima is growing thicker, and the fibrous elements are increased Simultaneously the lipoid is resorbed, and the histological picture suggests fibrosis

In human aortic atherosclerosis Anitschkow found both lipoid and fibrous plaques He held that the lipoid plaque was primary whereas the fibrous plaque was formed by resorption of the lipoid He concluded that atherosclerosis is an infiltrating rather than a degenerating process

From previous publications (3), as well as from the above study, it is assumable that the deposit of lipoid is a process which for some reason is connected to the intima even from infancy Gradually as the intima proliferates and shows incipient fibrosis, the lipoid will accumulate in lots, but up til now no reaction is visible in the surrounding connective tissue Only when the question is about large accumulations, a sclerosis is seen in the superficial intima, and then only in relation to a vigorous medial calcification

The actual fibrous intimal plaque—the primary sclerosis—occurs sporadically in the aorta at fairly large or small sections It is impossible to decide what causes this reaction in the intima, but it seems probable that it is a local ischemic reaction in the vascular wall which is badly supplied with oxygen beforehand Several animal experiments (2, 8, 10, 13) seem to indicate that anoxia plays a certain role in the formation of plaques

Blumenthal et al (4), in a similar study, found that in aortae from 3 young individuals with scanty contents of calcium in the media, there was a stronger medial calcification profoundly of the intimal plaques than beside them, he could not, however, rediscover this phenomenon in the remaining very large material

In the present material the author did not find that the content of calcium in the media was larger below the plaques than beside them The vigorous alteration in the intima corresponding to a fibrous plaque with formation of collagen-like, PAS-positive fibrils must probably be considered a reparative process on the part of the organism

The intimal reaction superficially of a lipoid plaque—secondary sclerosis—looks exactly like primary sclerosis, except that here it seems likely that the factor which causes the reaction is the lipoid accumulation As mentioned, a reaction in the intima superficially of a lipoid accumulation is only seen when the latter is sufficiently large, and when a vigorous medial calcification takes place simultaneously Whether the secondary sclerosis should be considered a reparative process superficially of the lipoid accumulation, so that the lipoid is but an indirect cause of the sclerosis, or whether the lipoid accumulation by its content of fatty acid and cholesterol crystals may have a stimulating effect on the formation of connective tissue, is impossible to decide Nor can the possibility be excluded that the lipoid plaque is formed from the fibrous plaque by a secondary interfibrillar accumulation of lipoid

The microscopical picture of the aorta from the patient with Muller-Harbitz's syndrome seems, however, to indicate that true lipoid plaques with superficial sclerosis are present

The shape of the fibril in the lipoid plaque is similar to that in the fibrous plaque Collagen fibrils do not react to Schiff's reagent after oxidation with periodic acid, whereas pre-collagen or pre-stages to collagen are PAS-positive, as they contain free 1 2 glycol-groups

(5, 6 7, 9) Judging from the histological picture the fibrils in the two plaque shapes are unripe collagen fibrils (precollagen) Whether the swollen fibril shape is formed by degeneration of the precollagen fibrils or by a "derailed' fibrillar synthesis cannot be decided The central uncoloured part of the fibrils consists presumably of degraded proteins, which as the fibrillar structure is obliterated forms an atheroma

As mentioned, it may be difficult macroscopically to distinguish between the lipoid accumulation and the actual fibrous plaque, and this may also apply to microscopical distinguishing In the case of a primary fibrous plaque without atheroma the picture is distinct, but immediately on appearing, the atheromas are seen to contain varying amounts of lipoid, depending on the other lipoid content of the intima On the other hand the secondary sclerosis above a lipoid accumulation may also present atheromatous parts Moreover, as mentioned, a deposit of calcium may take place in both shapes, which further veils the histological picture

In a previous publication *Bertelsen* (3) discussed the possibility that the calcification in the media is a crystallization of calcium salts in an organic matrix in relation to the collagen fibrils, and that chondroitin sulphate and other 'local factor('s)" play an important role in this process As mentioned, collagen like fibrils are seen before and during the calcification in the intima, and chondroitin sulphate is deposited among them The crystallization is distinctly seen to take place along the fibrils, and the theoretical reflections on the medial mineralization seem to apply completely to the intimal mineralization

Pernis et al (12) biochemically examined fibrous intimal plaques and macroscopically normal aortic tissue, and they found that both the content of collagen—expressed as hydroxyproline content—and the amount of hexose is considerably larger in the plaques than in the normal vascular tissue These results are in complete accordance with the above histological picture

According to the above, it is a question whether there is a clear distinction between the ordinary age-alterations and the alterations, related specially to the atherosclerotic process It seems more natural to consider the localized sclerotic process in the plaques as further development or finishing of the ordinary alteration, occurring in the aorta through life These alterations are necessary prerequisites of a plaque, this applies to secondary sclerosis with a lipoid accumulation as pathogenesis, as well as to primary sclerosis which is presumably based on the medial alterations

CONCLUSION AND SUMMARY

Ordinary ag...

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FROM THE RHEUMATIC RESEARCH LABORATORY (CHIEF G TEILUM M D), UNIVERSITY INSTITUTE OF PATHOLOGICAL ANATOMY COPENHAGEN DENMARK

HISTOCHEMICAL STUDIES ON ELASTIC MEMBRANES OF FETAL HUMAN AORTAE

By

J GARSJENSEN and SV BERTELSEN

Received 2 viii 60

Several authors are of the opinion that changes in the elastic membranes of the aorta play a prominent role, partly in the normal age-alterations of the vessel, and partly in the pathogenesis of arteroscleorosis (*Moon* (10), *Lansing* (9), *Gillman et al* (4))

For these reasons it is rather important that we should know the morphological and biochemical structure of the elastic membranes, there is however, still some uncertainty as regards these conditions

Gillman et al (4, 5)—in histochemical studies—has examined the

further found a PAS positive substance surrounding the elastic membranes

Hall et al (6)—in an electronmicroscopical study—investigated the morphology of elastin in the tunica media of human aortae He found membrane like structures consisting of differently calibrated fibres, partly ramified partly irregularly crossed to a network, in which the course of the fibres are proceeding in all directions The fibres are connected by an interfibrillar compact substance In places th...

... in close relation to the membranes Hall furthermore treated the elastin with elastase, and during the decomposition process he could establish an inner structure in the elastic fibre consisting of small fibrils, approximately 200 Å wide No periodicity was observed either in the large fibres or in the smaller fibrils

Several other investigators have demonstrated the inner fibrillar structure of the elastic fibres *Romhanyi* (12, 13), after examining the elastic membranes from human aortae by polarization microscopy,

We wish to thank J Malsen M D chief department of gynecology Copenhagen County Hospital Gentofte He most kindly placed all the specimens at our disposal

A distinction is made between lipoid plaques and fibrous plaques Both shapes are related to the intima without any actual alteration of the media, except the usual *age-alterations*

The media below the fibrous plaques always presents a vigorous mineralization in its entire thickness, whereas the lipoid plaques are often seen without any deposit of calcium in the media

The lipoid in the intima does not ordinarily influence the intimal connective tissue reaction Only when the question is about a medial mineralization and a very large accumulation of lipoid, a superficial *sclerosis with formation of fibrils* is observed

The fibrous plaque is a localized reparative process in the intima without any known cause The content of lipoid does not appear larger through the fibrous plaque than beside it

The relationship between the two plaque-shapes is discussed Possibly the fibrous plaque is primary, and the lipoid plaque is formed by deposit of lipoid among the fibrils

A case of Muller-Harbitz s syndrome shows, however, that primary lipoid plaques with superficial sclerosis of the intima are found

The deposit of calcium in the intimal plaques always take place interfibrillarly in the ground substance in close relation to the collagen like fibrils

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media) The outer layer which is somewhat narrower consists of connective tissue (the tunica adventitia) which is poorer in cells

In the media the cells are closely packed and the intercellular ground substance is observed only as a narrow zone In the ground substance a finely drawn fibrillar reticulation is visible the single fibres surrounding the cells (Figs 2 3) The fibres of the reticulation mentioned are argyrophile and PAS positive but no elastin coloured substance is visible at this stage The amorphous ground substance mentioned is Alcian blue positive and faintly metachromatic, but does not take stain by Schiff s reagent after oxidation with periodic acid

During *3rd fetal month* (Figs 4 6 8) the aortic wall grows moderately thicker the number of cells as well as the quantity of ground substance increasing Furthermore a considerable increase in the lumen of the vessel can be observed A cross section of the media shows that the cells are growing oblong and faintly S formed the longitudinal course running parallel to the lumen (Fig 4) The fibres of the above mentioned reticulation also become protracted so that at this stage we get an impression of tortuous fibrillar membranes concentric to the lumen here and there interconnected by fibres with a radial course A number of thin intertwining fibres constitute the individual "membrane both in silver staining and in PAS staining this phenomenon is most pronounced in silver staining (Fig 8) where as PAS staining shows a more diffuse somewhat blurred colouring of the fibres (Fig 6) The course of the membranes is *concentrical to* the lumen following the twistings of the cell nuclei one membrane outside the other along the whole width of the media

Throughout the fetal life the individual membranes increase in thickness as still more argyrophile fibres are formed and twined together with the other fibres (*Figs 7 8 9 14*) Gradually as the silver coloured membrane increases in thickness certain parts—appearing fragmentarily in the centre of the membrane —do not take the silver stain This phenomenon is observed first and most distinctly in the most luminal membrane (*Fig 14*) The colourless central parts gradually become fused so that all the membranes at partus appear with double contours in the silver staining (*Figs 15 17*)

Elastin coloured substance is not observed till about the middle of 3rd fetal month This substance appears as fragments which only gradually merge together forming continuous membranes which—similar to the membranes in silver and PAS staining—follow the tortuous cell nuclei concentrically with the lumen Continuous elastic membranes are not visible until about *4th fetal month* The elastic membranes increase in thickness with fetal age their course becoming at the same time less tortuous they seem gradually to straighten out as the lumen of the vessel is increasing (Figs 4 5 10 11)

Only one layer of cells separates the innermost membrane (the internal elastic membrane) from the lumen throughout the whole fetal

presumed that an inner structure of spirally coiled fibrils is conceivable Furthermore *Rhodin* (11)—*in an electronmicroscopical study*—showed that the elastic fibres in the trachea of the rat are composed of fine closely packed fibrils with a longitudinal course He described these fibrils as being 70–100 Å wide, and without periodicity The fibres are located in a homogenous non-fibrillar substance

Banga et al (1)and other investigators have examined the non-fibrillar putty substance These investigations showed that the putty substance contains acid mucopolysaccharides Banga further found certain *structural and biochemical similarities between elastic and collagen* fibres

Lansing et al (8) have repeatedly examined the biochemical composition of elastin They found that the amino-acid content increases with age, and that this increase especially applies to aspartic and glutamic acid, both of which contain free carboxyl-groups *Lansing* (8) held that the calcium which appears in the aorta with increasing age, was bound to the elastin

In the present study we have chosen to investigate the structure of the elastic membranes in human fetal aortae by histochemical methods hoping thereby to obtain a certain knowledge of their structural composition

MATERIALS AND METHODS

Aortae from 27 fetus The length of the fetus varies from 3 cm to 50 cm The fetal specimens were obtained at the gynaecological department of the Copenhagen County Hospital, Gentofte the majority of the fetus had been removed by hysterotomy others discharged after legal abortion The fetal specimens were immediately placed in refrigerator until preparation could take place

The length of the fetus in centimetre 3 4 5 5 6 5 7 7 8 9 5 10 10 11 13 14 14 16 18 19 20 21 21 25 27 27 32 49 50 50

The aortae were dissected and fixed partly in 4 per cent formaldehyde solution partly in 4 per cent basic lead acetate solution for 24 hours then dehydrated in alcohol and xylol and embedded in paraffin All sections were cut at 5 µ

The formalin fixed sections were stained in the following manner

(1) *Eskelund's* (3) modification of Gomori's aldehyde fuchsin staining
(2) Periodic acid Schiff's staining (PAS)
(3) PAS combined with Alcian blue staining (2)
(4) *Reticuline staining ad modum Foot*
(5) Hematoxylin eosin and van Gieson Hansen's stainings

The lead acetate fixed sections were stained with toluidine blue

Concerning toluidine blue staining and combined PAS Alcian blue see previous publications (2)

A few aortae were—after being dissected out hydrolyzed in 0 1 N NaOH at 98° C for 2 hours then fixed and embedded in paraffin in the usual manner

RESULTS

About the *2nd fetal month* two layers in the aortic wall can be differentiated out, *viz* a wide luminal and a narrow peripheral layer (Fig 1) Gross sections show that the luminal layer, being very rich in cells consists of 10 15 "rows of fairly large circular cells with circular nuclei arranged concentrically round the lumen (the tunica

media) The outer layer, which is somewhat narrower, consists of connective tissue (the tunica adventitia), which is poorer in cells

In the media the cells are closely packed, and the intercellular ground substance is observed only as a narrow zone In the ground substance a finely drawn fibrillar reticulation is visible, the single fibres surrounding the cells (Figs 2, 3) The fibres of the reticulation mentioned are argyrophile and PAS-positive, but no elastin-coloured substance is visible at this stage The amorphous *ground substance mentioned* is Alcian blue positive and faintly metachromatic, but does not take stain by Schiff's reagent after oxidation with periodic acid

During *3rd fetal month* (Figs 4, 6, 8) the aortic wall grows moderately thicker, the number of cells as well as the quantity of ground substance increasing Furthermore, a considerable increase in the lumen of the vessel can be observed A cross section of the media, shows that the cells are growing oblong and faintly S-formed, the longitudinal course running parallel to the lumen (Fig 4) The fibres of the above mentioned reticulation also become protracted, so that at this stage we get an impression of tortuous fibrillar membranes, concentric to the lumen, here and there interconnected by fibres with a radial course A number of thin intertwining fibres constitute the individual 'membrane' both in silver staining and in PAS-staining, this phenomenon is most pronounced in silver staining (Fig 8), whereas PAS staining shows a more diffuse, somewhat blurred colouring of the fibres (Fig 6) The course of the "membranes" is concentrical to the lumen, following the twistings of the cell nuclei, one "membrane" outside the other along the whole width of the media

Throughout the fetal life the individual "membranes" increase in thickness, as still more argyrophile fibres are formed and twined together with the other fibres (*Figs 3, 8, 9, 14*) *Gradually* as the silver-coloured "membrane" increases in thickness, certain parts—appearing fragmentarily in the centre of the "membrane"—do not take the silver stain This phenomenon is observed first and most distinctly in the most luminal "membrane" (*Fig 14*) The colourless central parts gradually become fused, so that all the "membranes" at partus appear with double contours in the silver staining (Figs 15, 17)

Elastin-coloured substance is not observed till about the middle of 3rd fetal month This *substance* appears as fragments, which only gradually merge together, forming continuous membranes which—similar to the "membranes" in silver and PAS staining—follow the tortuous cell nuclei concentrically with the lumen Continuous elastic membranes are not visible until about *4th fetal month* The elastic membranes increase in thickness with fetal age, their course becoming at the same time less *tortuous*, they seem gradually to straighten out as the lumen of the vessel is increasing (Figs 1, 5, 10, 11)

Only one layer of cells separates the innermost membrane (the internal elastic membrane) from the lumen throughout the whole fetal

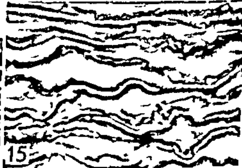

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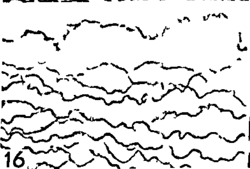

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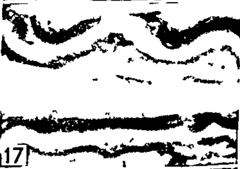

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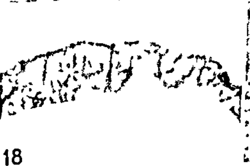

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directions (Fig 18) The elastin is specifically stained by elastin dye, whereas only a faint blacking of the membranes is obtained by PAS and silver stainings

DISCUSSION

The above results show that in the fetal human aortae we may find—even at a very early stage—an intercellular fibrillar structure located in a ground substance of acid mucopolysaccharides The fibres will take stain with silver and Schiff s reagent after oxidation with periodic acid, which seems to indicate that either the fibre itself, or a substance closely applied to it, contains 1, 2 glycol groups which are oxidated to aldehyde and or ketonic groups by periodic acid It is a well known fact that reticulin takes silver-stain, and is PAS positive, it is therefore a conclusion which suggests itself that the fibres mentioned are composed of reticulin

In the middle of 3rd fetal month fragments of elastin can be demonstrated which closely correspond to the above mentioned non-coloured parts, visible in the silver-stained membranes In the course of the 4th fetal month continuous membranes can be produced by elastin staining, this is in agreement with the fact that at this stage the silver stained elastic membranes appear double contoured with a continuous central brightening up

Krompecher (7) has examined the development of the elastic membranes in the subclavian artery in sheep fetus and found a similar fragmental origin of the elastic membranes, but he found the elastin-

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Fig 10 19

Fig 10 Aorta from a 27 cm long fetus (Eskelund s modification of Gomori s aldehyde fuchsin × 3o0) The internal elastic membrane appears now as a continuous membrane The other elastic membranes have a very regular course

Fig 11 Aorta from a 50 cm long fetus (Eskelund s modification of Gomori s aldehyde fuchsin × 460) A good deal of cells and ground substance among the finished membranes

Fig 12 As Fig 10 (PAS stain × 3o0) The internal elastic membrane is PAS pos The peripheral part of the membrane is most distinctly coloured

Fig 13 As Fig 11 (PAS Alcian blue × 140) The gr[illegible]nd s[illegible]bstance [illegible]

[illegible] coloured

Fig 1[illegible] Aorta from a 14 cm long fetus (Hydrolyzed with NaOH Eskelund s modificati[illegible]n of [illegible] [illegible]0) All the structures with the [illegible] obliterated

[illegible]40) A distinct mantle of argyro[illegible]

[illegible] (Hydrolyzed with NaOH Eskelund s modification of Gomori s aldehyde fuchsin × 1400) The membranes consist of differently calibrated fibres crossed to a netw[illegible]rk

Fig 19 Submicroscopic picture from media of a[illegible]rta from a 14 cm long fetus (× 6750) The grey amorph substance indicated with "F" are elastin In close relation to this very thin fibrilles with periodicity are seen

fragments at a somewhat earlier stage of the fetal life of the sheep He also observed that the elastic membranes developed in close relation to the tortuous medial cells which he named elastoblasts

It can be distinctly demonstrated that the fibres stained with silver and PAS are located morphologically in the same place as the membranes stained with elastin they all have a tortuous undulating course concentrically round the lumen The methods at hand will not permit us to decide whether argyrophile fibres form part of the elastic membrane as a constituent of the elastin or whether they are connected only with the surface of the elastin

The fact that elastin—after basic hydrolysis for 2 hours—is still specifically stained with elastin dye and even keeps its elasticity shows that it is an enormously resistant substance However all argyrophile fibres on the surface of the elastin are obliterated

Previous investigations have established the fact that in humans as well as in animals there is a close relationship between the reticular fibres and the elastin in the fully developed aortae (*Gillman et al* (4 5) *Bertelsen & Jensen* (2)) As already mentioned *Romhányi* (12 13) *Rhodin et al* (11) and *Hall et al* (6) have shown that elastin is composed of delicate fibrils contained in a non fibrillar substance

The above mentioned elastin structure seems to indicate that the argyrophile fibrillar substance observed may possibly form part of the structure of the elastic membranes as a constituent of elastin

With a view to further elucidating the above we have commenced an electronmicroscopical examination of human fetal aortae The result of these investigations will be published in a later study At present we shall only mention that so far we have found bunches of thin fibrils with periodicity located in close relation to the elastic substance and we have also observed a delicately drawn fibrillar structure *in the* elastin (Fig 19)

CONCLUSION

Argyrophile PAS positive fibres located in a ground substance of acid mucopolysaccharides are demonstrated in fetal human aortae as early as 2nd fetal month

Elastic fragments are demonstrated about the middle of 3rd fetal month and continuous elastic membranes are observed about 4th fetal month

A very close relationship between the argyrophile fibres and the elastin is demonstrated and it is discussed whether the fibres mentioned are likely to be component parts of the structure of the elastin in the elastic membranes

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EFFECT OF PROTEINS, FAT, CARBOHYDRATE AND SOME ENZYME INHIBITORS ON THE SYMPTOMS OF EXPERIMENTAL LATHYRISM

By

K JUVA, TAINA TUOMINEN, LEENA MIKKONEN and E KULONEN

Received 29 ix 60

Earlier feeding experiments in connection with the experimental lathyrism have yielded diverging results, partly because of an inaccurate assessment of the symptoms Therefore we repeated some of them using dietary additions of proteins, fat and carbohydrate with special emphasis on the roentgenological symptoms in bone Fresh approach was made with inhibitors of oxidative phosphorylation, of oxidation of the amines and of transamination The aggravating effect of isonicotinic acid hydrazides was a subject of a preliminary communication (*Juva, Mikkonen, Tuominen & Kulonen* 1959) and similar result was reported by *Roy, Lipton, Strong & Bird* (1959) It is suggested that when monoamine oxidase inhibitors like iproniazid are fed along with β-aminopropionitrile (BAPN) the detoxication is inhibited and higher effective concentrations of the β-aminopropionitrile result *in vivo*

Gelatin casein hydrolyzed casein and lactalbumin have all been reported to be protective when they comprise 20 40 per cent of the total food (*Dasler* 1954 1956) *Vivanco Jimenez Diaz & Palacios* (1951) come to the conclusion that animal protein contains some protective factor These opinions are not unanimous it is alleged that casein or animal proteins did not affect the development of osteolathyrism even in so high concentrations as 25 per cent casein of total diet (*Lewis & Esterer* 1943 *Ponseti Wawzonek Shepard Evans & Stearns* 1956) It is mentioned that the tendency to haemorrhage is increased on fat rich diets (*Barnett & Morgan* 1959) *Merkow Lipton Lalich & Strong* (1959) claim that the higher cyanoacetic acid excretion and minimal osseous deformities of rats on 24 per cent as compared with 12 per cent casein diet suggests that these animals were better able to detoxify BAPN by converting it to cyanoacetic acid

The suggested protective effect of proteins has prompted more detailed studies on almost all the amino acids Most attention has been paid to sulphur containing amino acids and glutamine Methionine is known to affect favorably the human neurolathyrism (*Rudra Chowdhury & Sinha* 1952) but most investigators have found no effect to the osteolathyrism (*Ponseti & Baird* 1952 *Bachhuber Lalich Angevine Schilling & Strong* 1955 *Dasler* 1956 *Barnett Bird Lalich & Strong* 1957) except on the condition caused by *Lathyrus pusillus* in the rat (*Lee Dupuy & Rolfs* 1956) *Dasler* (1956) has shown that both cysteine and glutamine (5 per cent) may delay the onset of the symptoms Following amino acids have been quite

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ineffective leucine (10 per cent), glycine (10 per cent), cystine (2 5 per cent) glutamic acid (10 per cent) histidine (5 per cent), aspartic acid (10 per cent), as

phas s has been put on antioxidants and *Ponseti et al* (1956) tested 13 aromatic antioxidants unsuccessfully In the list of other negative attempts may be mentioned

pyruvate (1 5 per cent) sodium propionate (5 per cent per cent) sodium th

of the skeletal pregnancy and protection afforded the fetus by estrone and progesterone when injected into the mother

Low doses of L *triiodothyronine* suppressed the mild forms of aminoacetonitrile *poisoning in rats* Extremely high doses of thyronine were required to accomplish the same result (*Ponseti* 1957)

EXPERIMENTAL

The *rats* were raised in our laboratory from Wistar stock and weighed 35–45 g in the beginning of the experiment (except in the nitrogen balance study) The sex was *not recorded* The litters were mixed when the experimental groups were formed The animals were k in their with the animals

X ray apparatu

The *basal dry diet* contained following ingredients

pea meal (either *Pisum sativum* or *Lathyrus odoratus*)	73,3 %
sucrose	11 7 %
	3 7 %
	3 7 %
	3 7 %
yeast	3 9 %

[1] Professors O *Järvi* and *Carl Wegelius* kindly placed the necessary equipment to our disposal and Dr *J Lindgren* gave much valuable instruction in its use

DEPARTMENT OF MEDICAL CHEMISTRY, UNIVERSITY OF TURKU TURKU 3 FINLAND

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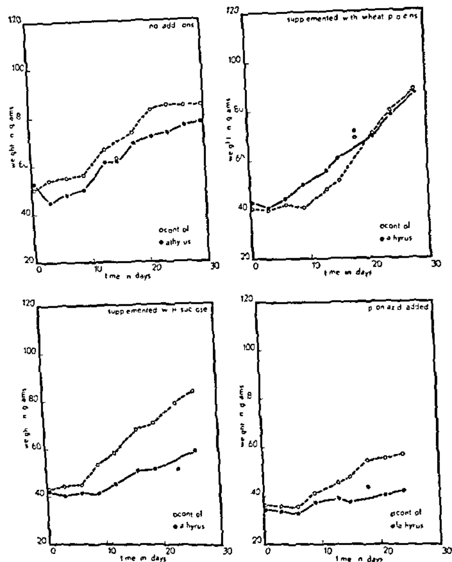


Fig 1
Samples of the growth curves. Each point represents means of 8 10 animals.
The composition of the diets is explained in the text

utilization the balance experiment described in Table 2 was carried out. The resorption of nitrogen is not impaired. The nitrogen balance was not negative during the *lathyrus*-diet, but on the return to the control diet. We think that our experiment is not conclusive as nitrogen balance is concerned.

All the animals in the *lathyrus* groups showed roentgenological signs of osteolathyrism: deformation of bones, especially of femur, callous

To this mixture was added (10 per cent v/w) following *oily solution*

vitamin A ("Arovit" Roche)	17 000 IU
vitamin D ("Calciferol" Ferrosan)	1 700 IU
vitamin K_1 (Merck)	0.85 g
vitamin E (Ph I grade)	0.85 g
olive oil (commercial)	1000 ml

Following *caloric additions* were used

sucrose 16.5 g to 100 g of basal diet,
gelatin (Merck, "golden label") 16.5 g to 100 g of basal diet,
fat (commercial olive oil) 16.5 g to 100 g of basal diet,
casein (purchased for rat feeding) 16.5 g to 100 g of basal diet,
wheat protein (obtained from wheat flour paste by exhaustive washing with tap water) 16.5 g to 100 g basal diet and
general enrichment (daily 1 g of fresh liver for every animal fresh cabbage *ad libitum and half of the oil ration replaced by cod liver oil)*

Following compositions were calculated for 100 g of the final diets using data from *Albritton* (1954)

Description	Protein	Carbohydrate	Lipid	Calories
basal diet	18.3 g	51.5 g	9.9 g	355 Cal
supplemented with sucrose	15.7 g	55.2 g	8.5 g	363 Cal
supplemented with proteins	30.0 g	40.9 g	8.5 g	347 Cal
supplemented with fat	17.0 g	48.0 g	16.3 g	394 Cal

The sweet pea seeds were from seed dealers old stock and contained only 6 mg% β aminopropionitrile (analyzed according *Garbut & Strong* 1957) This low value explains the comparative tardiness in the appearance of the symptoms of lathyrism

The following *non caloric additions* were used in *100 g of basal diet*

isopropylisoniazid (Marsilid Roche")	42 mg
isoniazid (Fluka)	20 mg
dinitrophenol (sodium salt Merck)	20 mg
pyridoxine (Ph I grade)	67 mg
desoxypyridoxine (Hoffman La Roche)	13.5 mg

Statistical evaluations were based on the *t* test Deviations were calculated by the standard methods

RESULTS

Caloric additions Examples on the growth curves are presented in Fig 1 The data on the weight gain are summarized in the Table Ia and its relation to the food consumption in Table Ib From Table Ia it is obvious that all the used proteins, gelatin, casein and gliadin have a protective effect Sucrose seems unfavourable fats and general improvement of the diet are without effect Data of individual animals are not available for statistical evaluation in the Table Ib However it seems that the *utilization of food for the growth* is decreased in the lathyritic groups and that protein supplement increases the utilization sucrose rather decreases it and fats are indifferent

Since it was not certain that the proteins in the sweet pea and the ordinary pea could not be different enough to cause this divergent

TABLE 1b

Correlation of Feed Consumption and Weight Gain in Rats on Lathyrus Diet with Various Supplements

Supplement	Food consumption (g day)		Weight gain in % of food consumption		
	Exp	Control	Exp	Control	Exp Control
None	6 2	6 3	14 4	19 4	0 67
None	7 5	8 1	16 1	26 7	
Caloric Supplements					
Sucrose	5 86	7 50	10 4	21 1	0 51
Sucrose	6 93	7 15	11 6	21 2	
Fat	7 33	7 86	15 9	24 9	0 64
Gelatin	5 62	5 95	16 4	18 7	0 88
Wheat protein	5 66	5 49	27 8	31 0	0 90
Casein	8 89	9 34	26 9	30 1	0 89
Liver and vegetables	9 02	10 90	20 1	26 4	0 76
Non Caloric Supplements					
Isopropylisoniazid	3 84	5 34	9 1	17.3	0 53
Isoniazid	5 67	7 45	14 6	25 7	0 53
Dinitrophenol	5 49	5 64	11 3	18 3	0 62
Desoxypyridoxin	5 47	6 43	14 6	24 8	0 59
Pyridoxin	5 44	6 48	15 6	24 0	0 65

insertions of tendons and degeneration of joints We believe that inspection and palpation do not give sufficient criteria on the advancement of lathyrism and that growth retardation and bone changes are not necessarily present simultaneously The bone changes were slightly alleviated in animals *receiving wheat protein* gelatin or sucrose

Addition of metabolic inhibitors X ray signs showed a bad aggravation by iproniazid and also by *isoniazid* Dinitrophenol pyridoxin and desoxypyridoxin were quite inert both on the X *ray symptoms* and weight gain It may be worthwhile to repeat the experiment with the desoxypyridoxine using higher doses of vitamin B antagonists

DISCUSSION

It is well established that proteins of very variable composition alleviate the effect of BAPN There seems *to be either metabolic* competition of β aminopropionitrile with the amino acids in general or some disturbance in the intermediary metabolism of amino acids caused by β aminopropionitrile Many of the interconversions of the amino acids or their ...
which however did r...
(1958) claim that th...
in lathyrism This could *not be* confirmed but the utilization of food is impaired otherwise A tempting speculation is that the formation of amino acids from carbohydrate skeletons is disturbed and that this defect is circumvented by the amino acid *abundance in the food*

TABLE 1a

Daily Weight Gain of Rats on Lathyrus Diets with Various Supplements

Supplement	No. of rats		Weight gain (g/day)			Relative weight gain	
	Exp	Contr	Exp	Contr	P	%*	P**
None	10	10	0 89 ± 058	1 22 ± 0 56	< 01	64 4 ± 7 8	(Reference)
None	10	10	1 21 ± 142	2 16 ± 146	< 001		
Caloric Supplements							
Sucrose	10	7	0 61 ± 061	1 59 ± 091	< 001	45 8 ± 7 3	< 0 10
Sucrose	10	10	0 81 ± 0 57	1 52 ± 085	< 001		
Fat	9	10	1 16 + 109	1 91 ± 103	< 001	C0 8 ± 11 1	
Gelatin	9	8	0 92 + 042	1 05 ± 141		87 6 ± 14 8	
Wheat protein	10	10	1 58 ± 074	1 70 ± 076		92 8 ± 6 5	< 0 01
Casein	10	10	2 39 ± 098	2 81 ± 088		84 8 ± 5 2	< 0 05
Liver and vegetables	10	10	1 82 ± 060	2 88 + 145	< 001	63 2 ± 6 0	
Non Caloric Supplements							
Isopropylisoniazid	10	10	0 35 + 038	0 92 ± 078	< 001	38 1 ± 13 8	< 0 10
Isoniazid	8	10	0 83 ± 121	1 92 + 089	< 001	43 0 ± 15 3	
Dinitrophenol	10	10	0 62 ± 077	1 03 ± 064	< 001	C0 2 ± 13 8	
Desoxypyridoxin	10	10	0 80 ± 056	1 60 + 067	< 001	50 0 ± 8 2	
Pyridoxin	10	9	0 85 + 068	1 56 ± 057	< 001	54 4 ± 8 8	

* Respective control diet as reference ** Respective basic diet as reference

The original working hypothesis was that the inhibition of the amino oxidase causes the increase of active β aminopropionitrile Whether this is the true mechanism of the iproniazid effect is doubtful Similar effect obtained with *isoniazid* which is only a weak amino oxidase inhibitor (*Pletscher* 1959), prompts a reference to the semicarbazides (*Dasler 1958*, *Millser & Dasler* 1959), which also cause lathyrism like changes The effects of these compounds on the intermediary metabolism are quite unknown, but they are all reactive with carbonyl groups

The findings of this work protection against lathyrism by the dietary proteins and its aggravation by hydrazides, apparently point to the metabolism of nitrogen and suggest a study on the metabolism of amino acids in BAPN-intoxication The effect of BAPN cannot be obtained locally (unpublished experiments by *J Viljanto*), which excludes a effect specific on fibroblasts The total incorporation of ^{14}C-proline into collagen was decreased both in carrageenin granuloma slices (*Kulonen, Salmi & Juva* 1960) and in chick embryos in the presence of BAPN (unpublished) However, there are observations on the incorporation of $^{15}NH_4Cl$ into the collagenous fractions of developing chick embryo (*Kulonen et al* 1960), which are difficult to reconcile with a decreased protein synthesis without assumption on some concomitant disturbance at the amino acid level

SUMMARY

The development of lathyrism in young rats was studied with diets supplemented with sucrose, fat various proteins, iproniazid *isoniazid*, dinitrophenol, pyridoxin or desoxypyridoxin Weight gain and roentgenological symptoms were used as criteria of the intoxication They did not run exactly in parallel

The intestinal was not nitrogen resorption of affected in lathyrism The ratio of food consumption and weight gain was decreased in all lathyritic groups

The protein supplements supported the weight gain on *lathyrus* diet and slightly alleviated the X ray symptoms Addition of iproniazid and isoniazid aggravated all the symptoms of lathyrism No clear effect was observed by the other additions

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TABLL 2

Nitrogen Balance on Lathyrus Diets

Variable	Control diet 1-5 days	*Lathyrus diet* 6-14 days	*Control diet* 15-19 days
Basic diet			
Weight of the rats g	216 (2nd day)	220 (13th day)	232 (19th day)
Food consumed g/day/rat	16.4	16.3	17.1
Nitrogen consumed mg/day/rat	554	630	576
Nitrogen excreted in feces mg/day/rat	163 (29.4 %)	145 (23.0 %)	154 (26.7 %)
Nitrogen excreted in urine mg/day/rat	345	411	528
Difference mg/day/rat	+46	+74	—126
Basic diet supplemented with sucrose			
Weight of the rats g	213 (2nd day)	219 (13th day)	233 (19th day)
Food consumed g/day/rat	16.2	15.9	15.9
Nitrogen consumed mg/day/rat	452	500	442
Nitrogen excreted in feces mg/day/rat	120 (26.5 %)	122 (24.4 %)	104 (23.6 %)
Nitrogen excreted in urine mg/day/rat	326	374	425
Difference mg/day/rat	+6	+4	—87

Figures present daily average of ten rats in each group

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IMMUNOCHEMICAL STUDIES ON SOME SEROLOGICAL CROSS REACTIONS IN THE KLEBSIELLA GROUP

3 *Further Studies on the Cross Reaction between Types A(1), E(5) and AE*

By

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with the technical assistance of *Unni Maini*

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In previous studies (1, 2) it was found that cross reactions between some strains of sero types A and AE appeared to have decreased in the course of time, and the possibility was suggested that this might be due to antigenic variations in one or more of these klebsiella strains It was also suggested that the presence of fucose in the molecule of the type AE capsular polysaccharide might be responsible for the cross reactivity with type A

The present paper is the result of a more extensive study of several strains of all the three types, which was carried out in order to clarify these questions

MATERIAL AND METHODS

The following strains were used

Type A Strain Sc one of Julianelle s original strains received from Dr *M C Morris* New York years ago and strains MA148 30228 1265 P32 and 27307 all isolated by the authors

Type AE Strains 047 085 0227 0282 0300 0314 0318 0325 0344 0365 0366 and 0380 all received from Amsterdam through the courtesy of Dr *W A Collier* and strain Sa7 isolated here

Type E Strains 025 026 027 and 0242 all [illegible]

[illegible]

[illegible] Professor Dr *W A Collier* Instituut voor Tropische Hygiene en Geographische Pathologie Amsterdam for sending us a collection of klebsiella strains

through glass filters G4 and G5 until most of the bacteria had been removed and finally the viscous slightly turbid suspension was filtered through Berkefeld N filter, resulting in a perfectly clear, viscous solution of polysaccharides, which are precipitated by addition of 3 volumes of ethanol and some sodium acetate. The mixture is left in the refrigerator until the following day, centrifuged in the cold washed three times with 96 per cent ethanol and dried in vacuo above P_2O_5.

Qualitative analysis of the composition of the polysaccharides was carried out by paper chromatography after hydrolysis with 3N H_2SO_4, neutralisation with $BaCO_3$ and evaporation to dryness in vacuo. The solvent used was ethyl acetate(5 vol.)-pyridin(2 vol.)-water(5 vol.). This system gave good separation of the monosaccharides in a short time.

A method described by *Ouchterlony* (3) was used for agar gel precipitation tests. Serum was placed in a central well in the agar plate, and antigens were distributed in 6 peripherical wells, arranged in a circle around the central well. The plates were kept at room temperature in a humid atmosphere and controlled daily until the precipitate patterns appeared to be fully developed. In some cases the wells were refilled when empty.

TABLE 1

Composition of the Capsular Polysaccharides of Klebsiella Strains

Sero type	Strains	Uronic acid	Galactose	Glucose	Mannose	Fucose	Per cent N
A	MA148, Sc 30228 1265, P32, 27307	+	+	+	0	+	0.5–2.2*
AE	O47, S57, O366	+	+	+	+	+	0.8–1.1
AE	O85, O227, O282, O300 O314 O318 O325 O344, O365, O380	+	+	+	+	0	0.5–1.1
E	O25, O26, O27, O242	+	+	+	+	0	0.5–1.1

* *Only one preparation contained as much* N *as* 2.2 per cent. All the others contained between 0.5 and 1.1 per cent.

RESULTS

Table 1 shows the composition of the capsular polysaccharides. All strains of type A contain 4 monosaccharides. The strains of type E also contain 4 sugars, but differ from type A in containing mannose instead of fucose. The strains of type AE, on the other hand fall into two groups. One group of 10 strains contain the same sugars as type E, whereas the second group of 3 strains contain fucose in addition. The previous statement that the type AE strain S 57 lacked glucose, must be modified, since chromatography of several later batches of this polysaccharide clearly showed the presence of this sugar. Whether this discrepancy is due to failure to detect glucose in the early attempts, possibly due to use of too small quantities, or to variation in the glucose content in the capsule of this strain, can not yet be ascertained. The earlier chromatograms have been rescrutinised, and we still fail to detect glucose. But this question will have to be left open until quantitative analyses of different batches of the polysaccharide have been carried out.

The nitrogen contents of polysaccharides prepared by the simplified method appear to be comparable to the contents of those prepared by the method of *Dudman & Wilkinson* (4) Only one of the preparations contained more than 1 1 per cent of N

TABLE 2

Capsular Reactions of Klebsiella Strains in Different Type-Specific Sera

Type	Strain	Immune-sera					
		Anti A MA 148	Anti AE S 57	Anti AE O366*	Anti AF O1	Anti AF O282*	Anti F O26
A	MA148	256	2	0	16	0	0
A	Sc	–	2	0	16	1(0)	1
AF	S57	4	32	64(32)	256	128	–
AF	O366	–	32	128	512	128	32
AF	O47		32	32	512	128	–
AF	O344	0	4	8(16)	32	32(64)	128
AE	O282	0	4	32(16)	64	128	–
AF	O325	0	4	8(16)	32	64	–
AF	O314	0	–	–		–	–
F	O25	0	–	32	64	128	–
E	O26			32(64)	–	256(128)	128
F	O27		4	32	–	256	–

Titers are given as the reciprocals of the highest serum dilutions giving positive reactions

* These titrations were repeated Figures in parentheses are the results of the second titrations when these differed from those of the first titration

– Test not done

In addition to the strains shown in the Table 6 Dutch strains (O85 O227 O300 O318 O365 and O380) and two Norwegian strains (3296 and 35538) failed to react in the available type A sera On the other hand strain O366 gave a positive reaction to dilution 1 2 in a different anti A serum

Results of titrations of the capsular reactions in different immune sera are shown in Table 2 In the anti-A serum shown in the table, which appeared to be highly type specific with little cross reacting antibody, only one AE-strain, the fucose-containing S 57, gave a positive reaction, whereas several of the fucose less strains and one E-strain failed to react In another anti-A serum, anti-Sc, the fucose-containing strain O 366 reacted to titer 1 2, whereas several fucose less strains failed to react Numerous tests with these strains in several anti A sera confirmed that the fucose-less strains either gave entirely negative reactions or that, in some instances, occasional positive cells [illegible] us (or in two phases), one possessing the cross-reacting antigen and the other lacking it In contrast in the fucose containing strains S 57 and O 366 all, or nearly all, organisms gave strongly positive reactions in the same sera

TABLE 3

Quantitative Precipitin Analyses with Immune Serum against Type AE Strain S57

Antigen S57a* AE		Antigen S57b* AE		Antigen O366 AE		Antigen O311 AE		Antigen O1[illegible] AE		Antigen O282 AE	
1	2	1	2	1	2	1	2	1	2	1	2
55	169	52	133	56	154	51	51	105	184	105	30
76	190	105	210	78	180	71	58	147	236	147	30
98	205	155	218	100	193	91	62	210	248	210	30
120	223	206	222	122	208	111	64	252	244	252	26
196	256	258	227	200	244	182	51	–	–	–	
240	244	–	–	244	244	–	–	–	–	–	

Antigen O27 E		Antigen MA 118 A		Antigen Sc A	
1	2	1	2	1	2
102	64	106	68	104	92
143	64	159	73	156	90
204	56	212	66	208	90
244	48	–		260	88

Columns marked 1 micrograms antigen added
Columns marked 2 micrograms antibody N precipitated
* Antigen $S57_a$ was prepared by the new simplified method antigen $S57_b$ was prepared by the method used by *Dudman & Wilkinson* by extraction with hot water
Serum was used in 1 ml volumes

The results obtained with the anti-AE sera show marked differences The sera against the fucose-containing strains, in particular anti S 57 and anti O 47, tend to distinguish rather sharply between the 3 fucose-containing strains on one side and the fucose-less AE-strains and the E-strains on the other With the serum anti O 282 against a fucose less strain, on the other hand, all AE and E strains gave nearly the same titers In the anti-E serum, finally, there was a 4-fold difference in titer between the fucose containing strains compared to a fucose less strain and a strain of type E

TABLE 4

Quantitative Precipitin Analyses with Immune Serum against Type E Strain O[illegible]

Antigen O[illegible]6 E		Antigen O366 AE		Antigen O311 AE		Antigen Sc A	
1	2	1	2	1	2	1	2
56	261	44	158	61	287	57	36
93	312	89	214	101	317	102	41
130	334	133	235	141	338	153	45
186	342	178	240	202	347	204	41
242	342	244	244	263	351		

Columns marked 1 micrograms antigen added
Columns marked 2 micrograms antibody N precipitated

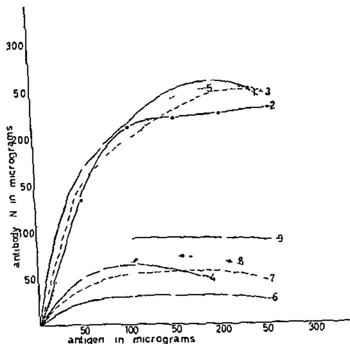


Fig 1

Immune serum ag

That cross reactivity between type A and type AE is not only dependent upon the fucose content, is shown by the fact that the serum against the fucose-positive strain O 366 lacks this antibody, whereas both the anti O 282 (fucose-less AE) and anti O 26(E) give weak positive reactions with one of the A-strains(Sc), like the serum anti O 25 used in the previous study (1)

Results of quantitative precipitin determinations with serum anti S 57 are shown in Table 3 and Fig 1 There is a very marked differentiation of the 4 fucose containing AE-strains, all of which precipitate nearly all of the antibody, from the rest of the antigens, which precipitate much less antibody Thus the fucose-less AE and the E antigens precipitate only between 12 and 25 per cent of the antibody, whereas two A-antigens precipitate 28 and 36 per cent This is surprising in view of the low titers these two strains gave in the same serum by the capsular reaction Two different preparations of the homologous antigen were used for comparison, one prepared by the hot water extraction method used by *Dudman & Wilkinson* (4) and the second by our simplified method There does not seem to be much difference

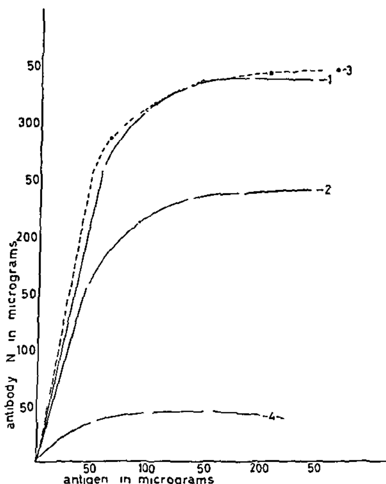


Fig 2

Immune serum against Klebsiella ozaenae type I strain O26 Antigens Curve 1 strain O26 type I —curve2 strain O366 type AI —curve 3 strain O344 type AI curve 4 strain Sc type A

between the two, although the one prepared by the simplified method may be slightly more reactive The individual variations between the antigens within each group are slight, but may possibly suggest some slight differences in the antigenic structure of even closely related strains

In view of the similarity between the fucose-less AE-antigens and the E-antigen and their differences from the fucose-positive AE antigens, tests were set up in the E-serum anti O 26 with the results shown in Table 4 and Fig 2 In this serum the E antigen and the fucose-less O 344-antigen show identical behaviour, whereas the fucose-positive O 366-antigen precipitates only 70 per cent of the antibody, and the A-antigen Sc precipitates about 13 per cent, which is again a surprising quantity in view of the weak capsular reaction

These results induced us to produce the two remaining sera, one against the fucose-less strain O 282 and the other against the fucose-positive O 366 The results appear in Tables 5 and 6 and Figs 3 and 4

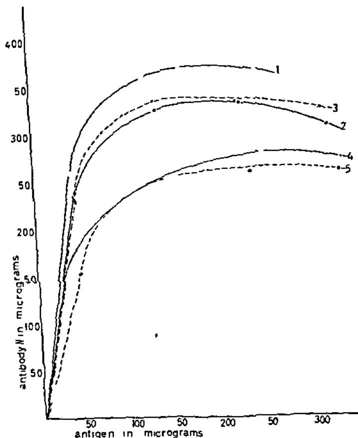


Fig 3

Table 5 and Fig 3 show that the homologous antigen removes sligh more antibody than any of the other antigens and therefore may h a certain degree of strain specificity, but the second fucose less antig O 344, and the E antigen, O 25, precipitate identical and nearly large amounts as the homolous antigen The fucose-containing tigens precipitate 70 75 per cent of the antibody The A antigen, did not produce measurable precipitate

With the serum anti O 366 (Table 6, Fig 4) the two fucose-cont ing antigens, O 366 and S 57, precipitate equal quantities of antib The E-antigen precipitates about 60 per cent of the antibody and two fucose less antigens, O 282 and O 344, precipitate only abou per cent

These results confirm that the fucose less AE-strains are very sin

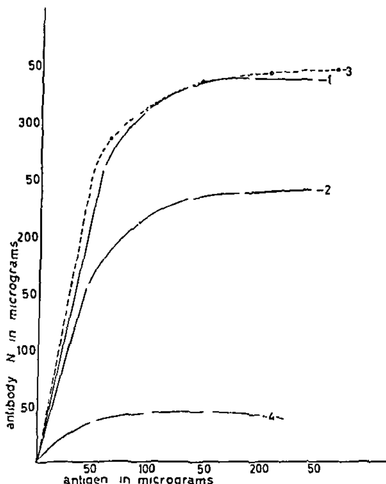


Fig 2

Immune serum against Klebsiella ozaenae type I strain O26 Antigens Curve 1 strain O26 type E—curve 2 strain O366 type AE —curve 3 strain O344 type AE curve 4 strain Se type A

between the two, although the one prepared by the simplified method may be slightly more reactive The individual variations between the antigens within each group are slight, but may possibly suggest some slight differences in the antigenic structure of even closely related strains

In view of the similarity between the fucose less AE-antigens and the E-antigen and their differences from the fucose-positive AE-antigens, tests were set up in the E-serum anti-O 26 with the results shown in Table 4 and Fig 2 In this serum the E-antigen and the fucose less O 344-antigen show identical behaviour whereas the fucose-positive O 366 antigen precipitates only 70 per cent of the antibody, and the A-antigen Se precipitates about 13 per cent, which is again a surprising quantity in view of the weak capsular reaction

These results induced us to produce the two remaining sera, one against the fucose-less strain O 282 and the other against the fucose-positive O 366 The results appear in Tables 5 and 6 and Figs 3 and 4

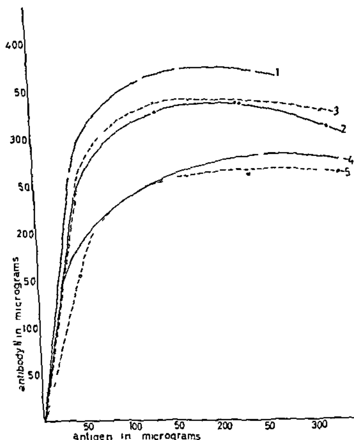


Fig 3

Table 5 and Fig 3 show that the homologous antigen removes slightly more antibody than any of the other antigens and therefore may have a certain degree of strain specificity, but the second fucose-less antigen, O 344 and the E-antigen, O 25, precipitate identical and nearly as large amounts as the homolous antigen The fucose containing antigens precipitate 70 75 per cent of the antibody The A-antigen, Sc, did not produce measurable precipitate

With the serum anti O 366 (Table 6, Fig 4) the two fucose-containing antigens, O 366 and S 57, precipitate equal quantities of antibody The L-antigen precipitates about 60 per cent of the antibody and the two fucose less antigens, O 282 and O 344, precipitate only about 50 per cent

These results confirm that the fucose-less AE-strains are very similar

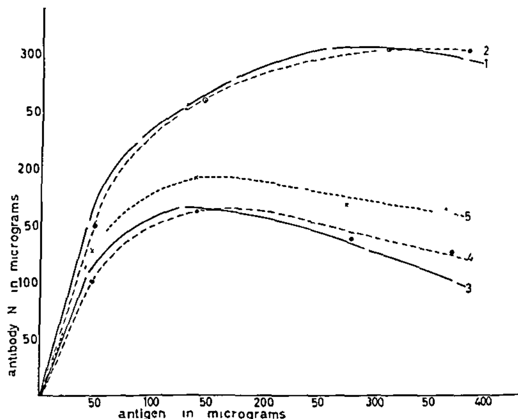


Fig 4

Immune serum against Klebsiella ozaenae type AE strain O366 Antigens curve 1 strain O366 type AE —curve 2 strain S57 type AE —curve 3 strain O282 type AE —curve 4 strain O344 type AE —curve 5 strain O25 type E

to each other and to type E, whereas the fucose-containing antigens behave nearly identically, but differ clearly from the fucose-less AE and E

The gel-precipitation tests with the anti-A serum (Figs 5 and 6) indicate that all the A-antigens are identical and that the technique used has not been sensitive enough to show the cross-reactions with the AE-antigens or the E-antigen

TABLE 5

Quantitative Precipitin Analyses with Immune Serum against Type AE Strain O282

Antigen 0282 AE		Antigen 0344 AE		Antigen 025 E		Antigen 0366 AE		Antigen S57 AE	
1	2	1	2	1	2	1	2	1	2
41	261	46	240	45	231	42	167	48	154
82	351	138	325	136	334	126	248	143	253
124	360	230	330	226	330	252	278	238	257
165	368	322	304	316	321	330	270	333	257
247	364	–							

Columns marked 1 micrograms antigen added
Columns marked 2 micrograms antibody N precipitated

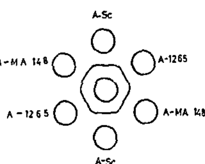


Fig 5
Immune serum anti A Sc in the central well

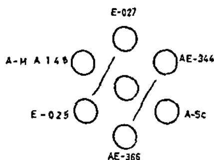


Fig 6
Same immune serum as in Fig 5

The results obtained with the serum against the fucose-less AE-strain O 282 (Figs 7, 7b, 7c, and 8) show that all antigens except the A-antigen Sc give double precipitate bands, lying closely together The antigen corresponding to the more central band appears to be common to all preparations, whereas the peripherical band shows reactions of identity between the fucose-less AE-antigens and the E antigen, but reactions of partial identity between these antigens and the fucose-containing AE-antigens Spurs are formed which show that the serum contains an antibody fraction which reacts with the former but not with the latter antigens This is in good agreement with the results of the quantitative precipitin tests (Fig 3)

TABLE 6

Quantitative Precipitin Analyses with Immune Serum against Type AE, Strain O366

Antigen 098 AE		Antigen Sa7 AE		Antigen 0282 AE		Antigen 0344 AE		Antigen 025 E	
1	2	1	2	1	2	1	2	1	2
42	150	48	150	41	107	46	101	45	128
84	227	143	261	124	167	138	163	136	193
126	253	306	308	289	128	276	150	271	170
168	278	381	308	371	103	368	128	362	167
[illegible]	308			–		–		–	–
378	300	–	–		–	–	–		–

Columns marked 1 micrograms antigen added
Columns marked 2 micrograms antibody [N] precipitated

The results of the tests with the serum anti-O 366 Figs 9 and 10) show that all antigens except the A-antigen produce two or three precipitate bands with this serum The bands produced by the fucose-containing antigens show identity reactions, likewise the bands produced by antigens O 344 and O 25 (fucose-less AE and E), and spurs are formed which show that the serum contains an antibody fraction which

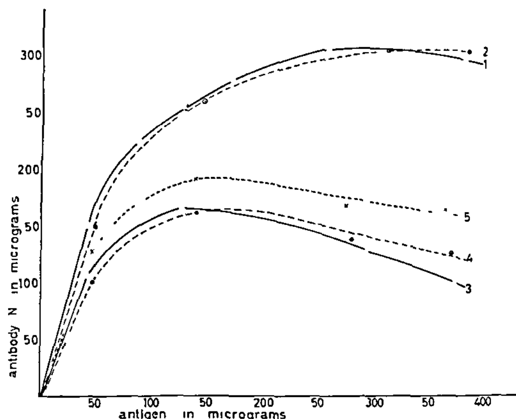


Fig 4

Immune serum against Klebsiella ozaenae type AF strain O366 Antigens curve 1 strain O366 type AF—curve 2 strain S57 type AE—curve 3 strain O282 type AF—curve 4 strain O344 type AF—curve 5 strain O25 type F

to each other and to type E, whereas the fucose-containing antigens behave nearly identically, but differ clearly from the fucose less AE and E

The gel-precipitation tests with the anti-A serum (Figs 5 and 6) indicate that all the A-antigens are identical and that the technique used has not been sensitive enough to show the cross-reactions with the AE-antigens or the E-antigen

TABLE 5

Quantitative Precipitin Analyses with Immune Serum against Type AF Strain O282

Antigen O282 AF		Antigen O344 AF		Antigen O25 F		Antigen O366 AF		Antigen S57 AE	
1	2	1	2	1	2	1	2	1	2
41	261	46	240	45	231	42	167	48	154
82	351	138	325	130	334	126	248	143	253
124	360	230	330	226	330	252	278	238	257
165	368	322	304	316	321	336	270	333	257
247	364	–	–						

Columns marked 1 micrograms antigen added
Columns marked 2 micrograms antibody N precipitated

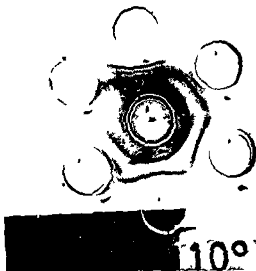


Fig 7 c
Photograph correspond ng to the drawing in Fig 7 The photograph and drawing were made after about 3 weeks at room temperature The wells had been refilled once at about the middle of the incubation period

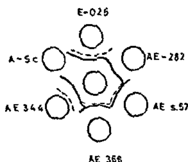


Fig 9
Immune serum anti AE O366 in the central well

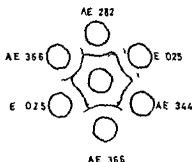


Fig 10
Same immune serum as in Fig 9

DISCUSSION

As already stated previous studies led us to suspect that *some* Klebsiella strains might undergo antigenic variation It was found that different AE strains varied in their reactivity in anti A sera and one strain (O 314) which failed to react was found to lack fucose *in the* capsular polysaccharide It was therefore suggested that the presence

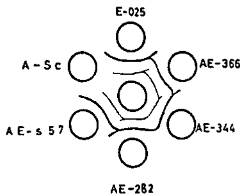


Fig 7
Immune serum anti AI 0282 in the central well

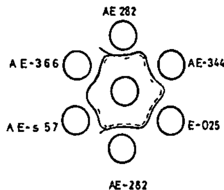


Fig 8
Same immune serum as in Figs 7 7 b and 7 c

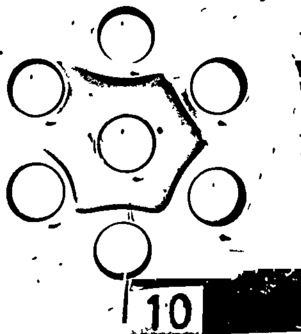


Fig 7 b
The same arrangement as in Fig 7 The photo was taken after about 1 week at room temperature and after only one filling of the wells

does not react with the latter antigens, but some of the bands are too faint and too short for interpretation It is possible that other arrangements of the wells may give more instructive results

Figs 11, 11b, and 12 show the reactions obtained with serum anti O 47, an old serum which was only available in small quantity The results agree with those obtained with serum anti O 366

In the drawings, Figs 5 to 12, heavy, whole lines indicate sharp and easily visible precipitates, light and broken lines indicate weak and indistinct precipitates

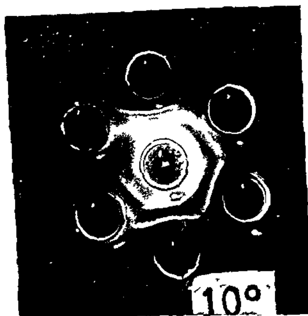


Fig 7c
Photograph corresponding to the drawing in Fig 7 The photograph and drawing were made after about 3 weeks at room temperature The wells had been refilled once at about the middle of the incubation period

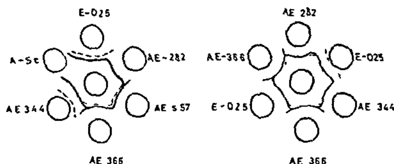


Fig 9
Immune serum anti AE O366 in the central well

Fig 10
Same immune serum as in Fig 9

DISCUSSION

As already stated previous studies led us to suspect that some *Klebsiella* strains might undergo antigenic variation It was found that different AE strains varied in their reactivity in anti A sera, and one *strain (O 314)* which failed to react, was found to lack fucose in the capsular polysaccharide It was therefore suggested that the presence

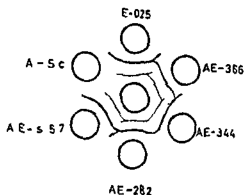


Fig 7
Immune serum anti-AE 0282 in the central well

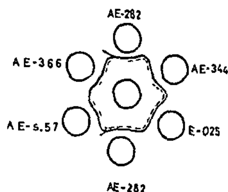


Fig 8
Same immune serum as in Figs 7, 7 b and 7 c

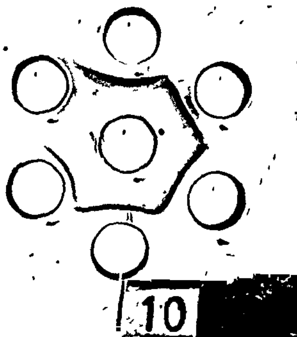


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In the drawings, Figs 5 to 12, heavy whole lines indicate sharp and easily visible precipitates, light and broken lines indicate weak and indistinct precipitates

But fortunately one of the 10 new Dutch strains was found to react in our anti A sera, and when we found that still another strain, the strain O 47 which had been received from Amsterdam years ago, also reacted, and that these three strains contained fucose in contrast to all the other strains, the chances of proving our hypotheses appeared brighter Nevertheless, we are not yet in a position to produce conclusive proof

The apparent loss or reduction of cross reactivity in anti-A sera might be explained in different ways Possibly it might be due to a change of the A-strains with loss of some of the antigenic structures responsible for the cross-reactivity with AE The difficulties we have encountered in producing anti-A sera with a satisfactory level of antibody cross reactive with the fucose-containing AE-strains, might speak in favour of this Since this would mean that the several different A strains used for immunisation must have changed in the same manner, this explanation may appear unlikely, especially so because some of these A strains were old laboratory strains long before this study was started

Or the explanation might be sought in the variability of the antibody response of individual rabbits to the same antigens It might conceivably be due either to genetic differences between the rabbits, or just to bad luck, that we had failed to produce sera of the same apparent quality as in former years While there is no doubt that the individual variability of rabbit sera is a factor of great importance in the study of serological cross-reactions, it seems improbable that this could be the explanation of our findings Also the difference between the two sub groups of the AE-strains would remain unexplained

It seems to us that the most reasonable hypothesis by far is the one we started with, that the explanation is an antigenic variation of the AE-strains, involving loss of fucose and simultaneous loss or reduction of cross-reactivity with type A It is a fact that all the AE-strains originally were identified as such by their characteristic reactions in anti-A, anti F and anti-AE sera, and it is obvious that their reactions at that time must have differentiated them clearly from type E In support of this it may be mentioned that in our limited experience the typing of AE- and E strains has caused no difficulty at all It is equally certain that by means of the sera now available to us and by chemical analysis only 3 of the strains could have been identified as type AE to-day The rest of the strains would have had to be classified as type E

Whether this variation is the result of mutation or of some kind of phase variation possibly of a similar nature as various kinds of phase variation of Salmonella antigens, is a matter of conjecture In the latter case it would be reasonable to expect the phenomenon to be reversible, and that the change in either direction should be producible by changing the cultural conditions We have ... out numerous ... the media, the ... freeze-drying

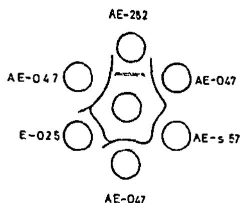


Fig 11
Immune serum anti AE 047 in the central well

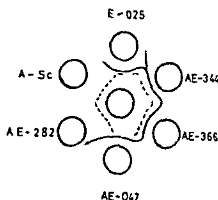


Fig 12
Same immune serum as in Figs 11 and 11 b

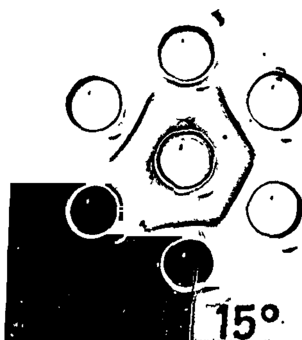


Fig 11 b
Photographic corresponding to the drawing in Fig 11

or absence of fucose might have an influence on the cross-reactivity in anti-A serum In order to test these hypotheses we asked for and received a number of additional strains of sero-types AE and E

It was a rather disturbing experience to test these strains One strain after the other failed to react in type A serum, indeed of the first 7 strains tested not a single one reacted At first this was very puzzling and led us to suspect that it must be our A-strains which had changed, and that our strain S 57, which did react in the A-sera, must be atypical

a freshly isolated strain is desirable but we have not yet had the luck of encountering such a strain in the course of our study

The results of the gel precipitation tests where double or triple precipitate lines were produced by some systems suggest that these serological systems may be more complicated than expected but our work along these lines does not yet seem to have advanced far enough for more detailed discussion

Many attempts have been made to study the role of the individual monosaccharides contained in the capsular polysaccharides as determinants of serologial specificity Thus we have tried the inhibition method used by *Staub & Tinelli* (5) as well as their method of mild oxydation of the polysaccharides but so far without much success Our results have mostly been negative but this work is being continued and will be reported in a subsequent paper

SUMMARY

A number of Klebsiella strains of sero types A(1) AE and E(5) were studied The capsular polysaccharides of all strains of types A and E had the composition previously reported The polysaccharides from the strains of type AE on the other hand were of two kinds one isolated from 10 strains containing the same sugars as the type E polysaccharide the other isolated from 3 strains containing fucose in addition like the type A polysaccharide Only the latter group of strains cross reacted with type A in the manner characteristic of type AE whereas the serological specificity of the former group was identical or closely related to that of type E

This difference between two groups of strains is thought to be due to a mutation or phase variation involving loss of fucose and simultaneous reduction of cross reactivity with type A but attempts to bring about this change in either direction artificially failed

Cross reactions between these three sero types are of two kinds one between fucose containing strains of type AE and type A and presumably due to fucose-containing structures and the other between type A and type E as well as fucose less strains derived from type AE The chemical basis of the latter cross reaction is not yet known nor is it known whether the corresponding antibody also reacts with the fucose containing AE strains The loss of fucose not only involves loss of a part of the specificity characteristic of type AE but also a gain of new specificity that characteristic of type E

Results of double diffusion tests in agar on the whole were in good agreement with those obtained by quantitative precipitin analysis but in addition indicated that some of the serological systems studied may be complex

was also tested, since it seemed possible that the Dutch strains might have undergone the change while in the lyophile state or in the course of lyophilisation But all our attemps so far have failed Our findings are therefore also compatible with the idea that the change is caused by mutation and a selective influence of the cultural conditions in favour of the fucose-less mutant However, it is puzzling that even freshly isolated strains can easily be identified as "pure" E-type, even by typing the primary culture (we recently encountered such a strain), and also that some strains retain the AE-character long after other strains have changed

The possibility that the action of phage could be behind the change as in certain antigenic variations in Salmonella, will be explored

In any case our results suggest that the types AE and E are only different mutants or phases of the same type, and that our "degraded" AE-strains have actually changed to E

The described change probably involves loss of the antigenic structures which are responsible for the marked tendency to cross-react with type A, that is the special characteristic of type AE It is highly probable that fucose is an essential element of those structures

The fact that cross-reactions, usually only of very low titer, between types A and E can be demonstrated with certain sera, shows that these fucose-containing structures are not the only basis of cross-reactivity There is not evidence to show what is the basis of this second cross reaction, but the fact that these types have several monosaccharides in common opens many possibilities

The difference between the fucose-containing and fucose-less strains can not be explained only as a loss of one part of the specificity or of one antigenic factor There is also a gain in specificity shown by the fact that immune sera against the fucose-less strains as well as against E-strains contain antibody fractions which do not react with the fucose-containing antigens This may mean that previously hidden antigenic structures are uncovered by the loss of fucose or that entirely new structures are developed instead of those that have been lost Further quantitative studies of the chemical composition of the capsular polysaccharides might help in studying this problem

Although the results reported in this study go far to explain the variable reactions of the AE-strains, we are not quite satified that it is the whole explanation We still have an impression, which may be wrong, that it is more difficult to obtain anti A sera which give satisfactory cross-reactions with the undegraded type AE, than it used to be This may be due to chance only, but we have also considered the possibility that the change from typical AE to "pure" E may be a gradual process, either with a slow gradual change of the composition of each individual capsule or by a gradual selection from a heterogeneous bacterial population If so, even our fucose containing strains might be "degraded" compared to freshly isolated strains A comparison with

FROM THE UNIVERSITY OF BERGEN SCHOOL OF MEDICINE MEDICAL DEPARTMENT B AND THE GADE INSTITUTE DEPARTMENT OF MICROBIOLOGY BERGEN NORWAY

THE EFFECT OF BROAD SPECTRUM ANTIBIOTICS ON THE FAECAL STAPHYLOCOCCAL AND MONILIAL FLORA IN MAN

By

T HOFSTAD and A WORMNES

Received 13 ix 60

It is common knowledge that the tetracycline antibiotics and to some extent chloramphenicol when given orally may give rise to a marked alteration in the faecal flora Susceptible organisms *i e* the gram negative population are suppressed while others such as *Candida albicans* and particularly *Staphylococcus aureus* tend to proliferate

In the present study the faecal staphylococcal flora was investigated before and after administration of tetracyclines and chloramphenicol A few patients were treated with erythromycin and a selected group of patients was given tetracycline oleandomycin At the same time the occurrence in the stools of *Candida albicans* was studied

MATERIALS

METHODS

The pathogenic staphylococci were isolated from phenol red mannitol agar plates (4) after incubation at 37° C for 48 hours The production of free coagulase was used as the only criterion of pathogenicity The sensitivity of the pathogenic strains was determined according to the disc method of Ericsson *et al* (5)

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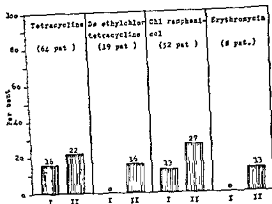


Fig 2
The occurrence of *Candida albicans* in the stools before (I) and after (II) treatment with broad spectrum antibiotics

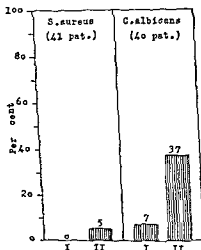


Fig 3
The occurrence of *Staphylococcus aureus* and *Candida albicans* in the stools before (I) and after (II) administration of oleandomycin tetracycline

(Fig 3) The medication induced a rise in the frequency of *Candida albicans* from seven to 37 per cent

The only side effects observed were diarrhoea and pruritus ani (Table 1) The diarrhoea which in most cases was accompanied by some nausea and general malaise was mild and ceased when medication was stopped Pathogenic staphylococci were found in the stools of one of the patients with diarrhoea and both patients complaining of pruritus ani had positive stool cultures for *Candida albicans* The side effects appeared after one to 14 days of medication

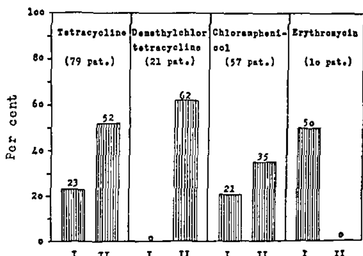


Fig 1
The occurrence of *Staphylococcus aureus* in the stools before (I) and after (II) treatment with broad spectrum antibiotics

For the isolation of *Candida albicans* the stools were incubated on Bacto Chlamydospore Agar (Difco code No 0513), at room temperature The plates were examined twice weekly for up to four weeks The final identification was made by direct microscopical examination of suspect colonies on the plates and by microscopical examination of the colonies mounted wet in lactophenol cotton blue

RESULTS

Series 1 Following the treatment with antibiotics there was an average rise in the frequency of pathogenic staphylococci in the stools from 21 to 44 per cent (Fig 1) The greatest increase was seen among the patients receiving demethylchlortetracycline Ten patients who were all treated with erythromycin because of some staphylococcal infection, had negative stool cultures for pathogenic staphylococci on cessation of treatment The staphylococci which persisted or appeared in the stools during treatment were in all instances resistant or only slightly sensitive to the antibiotic given

The appearance of pathogenic staphylococci in the stools was accompanied by diarrhoea in eight patients Four of these received tetracycline, two received demethylchlortetracycline, while chloramphenicol had been given to the remaining two patients One of them had numerous watery stools and had to be treated with erythromycin

The percentage of *Candida albicans* in the stools rose from 12 to 22 following the antibiotic treatment (Fig 2)

The records of nearly two thirds of the patients in this series gave no information on previous treatment with other antimicrobials These patients were analysed as a separate group The results obtained were however, quite analogous to those for the whole series

Series 2 Pathogenic staphylococci appeared in the stools of two patients during the administration of tetracycline-oleandomycin

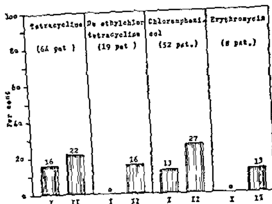


Fig 2
The occurrence of *Candida albicans* in the stools before (I) and after (II) treatment with broad spectrum antibiotics

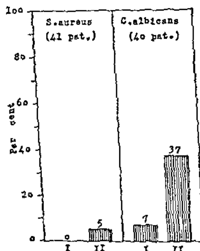


Fig 3
The occurrence of *Staphylococcus aureus* and *Candida albicans* in the stools before (I) and after (II) administration of oleandomycin tetracycline

(*Fig* 3) The medication induced a rise in the frequency of *Candida albicans* from seven to 37 per cent

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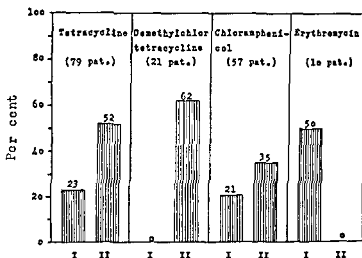


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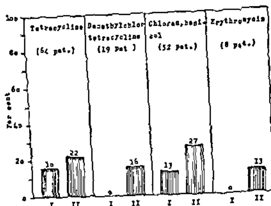


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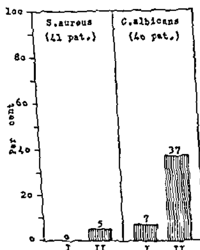


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TABLE 1

Side Effects Attributable to Administration of Tetracycline Oleandomycin (Sigmamycin)

Dosage schedules	Total no patients	Side effects	
		Diarrhoea	*Pruritus ani*
0 25 × 4 +	26	3	2
0 50 × 4	15	3	–
Total	41	6	2

+ 0 50 × 4 initially

DISCUSSION

The average occurrence of pathogenic staphylococci in the stools before treatment in 21 per cent of the patients in series 1 is somewhat higher than reported from other hospital wards (1 3 6 7) None of the "healthy" patients in series 2 had, on the other hand, positive stool cultures for pathogenic staphylococci before the administration of antibiotics, *i e* on- or shortly after admission These results seem to indicate that pathogenic staphylococci are not normally, or only infrequently, present in the stools of healthy individuals outside hospitals Similar findings have been reported by *Cabrera et al* (2)

The highest percentages of pathogenic staphylococci in the stools were found in patients taking tetracyclines *Welch et al* (9) in a nationwide survey from more than 800 hospitals in the United States, found that these antibiotics accounted for most cases of severe secondary staphylococcal infections

The different figures in the tetracycline- and demethylchlortetracycline group are difficult to explain They may depend on the fact that demethylchlortetracycline was given over a period of only six months when the department was more than usually contaminated with pathogenic staphylococci The patients who developed positive stool cultures for pathogenic staphylococci during treatment with demethylchlortetracycline had on the other hand, been hospitalized for an average period of seven days before the antibiotic treatment started

Erythromycin is still a potent antistaphylococcal agent in our hospital

The antibiotic combination tetracycline oleandomycin was well tolerated and no serious side effects were observed The combination *did* not however prevent the invasion of the bowel by pathogenic staphylococci

No serious adverse effects attributable to overgrowth of *Candida albicans* were seen in our two series, and the rise in the percentage of this microorganism following the administration of antibiotics was moderate *Welch et al* in their survey report only eight cases of severe

moniliasis among nearly 200,000 hospital patients treated with antibiotics It seems therefore, as pointed out by *Robinson* (8) that the sporadic cases of moniliasis which have been reported following treatment with broad spectrum antibiotics, are due more to the underlying wasting disease with lowered resistance to invasion of *Candida albicans* than to the antibiotic therapy

SUMMARY

The occurrence of *Staphylococcus aureus* and *Candida albicans* in the stools before and after administration of broad spectrum antibiotics was studied in two series of patients The first series comprised 167 patients suffering from various infectious diseases who were treated with tetracycline, demethylchlortetracycline, chloramphenicol or erythromycin The other series consisted of 41 otherwise healthy patients with peptic ulcer to whom tetracycline-oleandomycin was administered

The percentage of pathogenic staphylococci rose from 21 to 44 in the first series The greatest increase was seen after treatment with demethylchlortetracycline Diarrhoea was observed in eight patients

Pathogenic staphylococci appeared in the stools of two of the patients in the other series following the medication The medication was discontinued in six patients because of loose stools

The rise in the percentage of *Candida albicans* in both series was moderate and without clinical significance

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FROM DEPARTMENT OF GENETICS, UNIVERSITY OF WISCONSIN, MADISON, WIS. AND THE INTERNATIONAL SALMONELLA & ESCHERICHIA CENTRE, STATENS SERUMINSTITUT, COPENHAGEN, DENMARK

THE FERTILITY OF ESCHERICHIA COLI ANTIGEN TEST STRAINS IN CROSSES WITH K 12

By

FRITS ØRSKOV and IDA ØRSKOV

Received 5.xii.60

Sexual recombination in *Escherichia coli* has been studied almost exclusively with strain "K 12". The initial choice of this strain was entirely fortuitous (*Lederberg & Lederberg* 1956, *Gray & Tatum* 1944). While many aspects of the life cycle have been successfully analyzed with this particular strain, at least two considerations have motivated a search for the distribution of sexual interfertility among a wider group of strains: (1) the interest in the status of the *Escherichia coli* group, as a gene pool and (2) the possibility that some genetic differences not evident from mutation in the laboratory might be found among diverse strains from natural habitats. In the latter category, the most interesting features might well involve the mechanism of sexual compatibility among various serotypes.

A preliminary screening of wild type strains seemed to justify these expectations (*Lederberg* 1951). This study was however conducted before the Hfr, F+, F− system of sexual compatibility was adequately understood (*Cavalli* 1950, *Lederberg, Cavalli & Lederberg* 1953, *Hayes* 1953). In view of the promise of immunogenetic factors in future work it was, therefore, decided to take advantage of the collection of established serotypes of *Escherichia coli* for a review of genetic interactions in the group. Strain "K 12" itself poses formidable difficulties for immunological studies in account of its virtual loss of O and K antigens, the strain having been cultivated for other purposes and without special scrutiny for its serological properties since 1922. The establishment of a serological scheme for *E. coli* (*Kauffmann* 1954, *Ewing* 1956) indeed represents a substantial investment that can be exploited in further genotypic analysis of the species.

These studies were conducted for the most part at the *Department of Genetics, University of Wisconsin, Madison, Wis.* The work was supported by research grants (to Professor Lederberg) from the *National Science Foundation* and from the *National Cancer Institute* (C 2157), *U. S. Public Health Service*. *I. Ørskov* was a fellow of *the American Association for University Women*.

The E coli Antigenic Scheme

Three main types of antigens are depicted in the *E coli* group (1) The O antigens which are thermostable lipopolysaccharide complexes constituting part of the cell wall (2) the K antigens which are envelope or capsule antigens of polysaccharide nature, historically three types of K antigens A, B and L have been described They are differentiated by the varying thermostability of their agglutinability, their capacity to evoke the formation of agglutinins and their agglutinin binding capacity (3) The H or flagellar antigens, by contrast with *Salmonella*, which frequently display alternative flagellar forms only monophasic strains have been found so far in the coli group The first antigenic scheme for the *E coli* group was published by *Kauffmann* in 1944 This scheme contained 20 different O groups, 17 K antigens and 3 H antigens Since then the scheme has been steadily extended and it comprised when the present examinations were carried out

137 O antigens
80 K antigens
43 H antigens

corresponding to a somewhat smaller number of type strains, since some of the K and H type strains are also represented in the O series The serotype of a coli strain is given as follows O 111 K 58 H 2[1] The O K and H antigens exist in a great number of different combinations, if they could recombine freely the absolute number of coli serotypes containing the known antigens would be close to half a million In practice the number is reduced, as it has been observed that a number of O and K antigens (especially OA and OB) are closely connected For example it could be mentioned that the combinations O 55 K 59 and O 111 K 58 are known only in the indicated pairs On the other hand that the H antigens can be combined freely with diverse OK groups is illustrated by the experience from the O 55 K 59 and O 111 K 58 groups Among the rather limited number of these strains whose H antigens have been recorded at least 8 different H antigen combinations for O 111 K 58 and 10 for O 55 K 59 have been detected (*Ewing* 1956 *Orskov* 1956)

Sexual Compatibility in E coli

Sexual recombination in *E coli* is mediated by contact between cells of different mating type In order to give a productive cross one type of cells (F^+) acts as genetic donor or male and the other as recipient or female (F^-) However, F^+ are ambivalent and $F^+ \times F^+$ crosses give a few recombinants Most of the K 12 strains are F^+ upon mixing with "K 12 F" culture they exhibit a low frequency of recombination

[1] According to earlier convention this would have been designated O111 B4 H2 the notations here follows *Kauffmann Orskov & Ewing* (1956)

(10^{-5} or less expressed as the observed ratio of recombinant to input parental cells) Strains termed Hfr (*Cavalli* 1950, *Hayes* 1953) show a high frequency of recombination in crosses with F⁻ strains (10^{-1} to 10^{-2}) In most sexual crossing experiments carried out so far both parent strains have been auxotrophic mutants which were unable to synthesise one or more compounds necessary for growth, most often amino acids This procedure requires considerable handling of each parent strain to produce the necessary mutants To simplify the screening of a large number of strains the so-called SRP-technique (*streptomycin-resistance-prototroph*) was developed (*Lederberg* 1951) Recombinants are selected from test crosses between auxotrophic, streptomycin-resistant tester strains and various prototrophic, streptomycin-sensitive *i e* wild type strains Thus a large number of *coli* wild type strains could be screened both for fertility and for mating type

METHODS

The parent strains were grown in penassay broth Difco for 20 hours and 0 5 ml from each parent culture was inoculated into a fresh broth The mixed culture was incubated for another 20 hours centrifuged and the pellet resuspended in 0 5 ml distilled water One drop of this suspension was spread onto minimal medium (see below) Controls were provided by plating the washed parent cultures onto the same medium as used for crosses The plates were read after 48 hours incubation at 37° C No further analysis of the recombinant colonies was carried out (*Lederberg* 1951)

Media For fluid medium penassay broth Difco was most often used In some cases autoclaved ox heart infusion broth produced in Statens Seruminstitut Copenhagen was employed No difference as to the usefulness of these two media could be found EMS agar plates (*Lederberg* 1950) supplemented with dihydrostreptomycin 100 µg/ml and galactose 1 per cent was used as selective medium for the recombinants

Strains All type strains for coli antigens which were established up till 1957 were examined

O antigen test strains	137
K antigen test strains	80
H antigen test strains	43

Two of the O type strains were found to be streptomycin resistant (O126 and O127) four O type strains have previously been found not to belong to *E coli* but to the *Citrobacter* group (*Ørskov* 1956) and finally O47 has been lost many years ago Of the K type strains 29 were identical with different O type strains Among the 43 H antigen type strains 23 were already represented among the O or K type strains two H type strains have been found to belong the *Citrobacter* group The reduced number of different type strains examined was therefore

O antigen test strains	130
K antigen test strains	51
H antigen test sttrains	18
Total	199

76 *E coli* strains belonging to the OK group O26 K60 O55 K59 and O111 K58 in many different H antigen combinations were further examined (Table 1)

72 *Klebsiella* type strains representing the same number of different capsule antigens were also included

The auxotrophic coli strains with known fertility used in the crosses can be found in Table 2 These last mentioned strains were all developed in Dept of Genetics University of Wisconsin Madison Wisconsin U S A

TABLE 1

E. coli Strains Belonging to the OK Types O26 K60, O55 K59 and O111 K58 Tested

Serotype antigens O	K	H	Number of strains
26	60	–	7
26	60	8	1
26	60	11	5
26	60	32	3
55	59	–	8
55	59	2	3
55	59	4	2
55	59	6	3
55	59	7	4
55	59	8	3
55	59	10	2
55	59	11	2
55	59	16	1
55	59	21	1
55	59	25	1
55	59	27	3
55	59	32	3
55	59	34	1
111	58		6
111	58	2	4
111	58	4	2
111	58	11	1
111	58	12	1
111	58	16	1
111	58	21	1
111	58	27	1
Totally			76

TABLE 2

Auxotrophic Strains Used

Source	Strain designation	Markers	Serotype
K12	W1607	F M Sr	O K? H48
	W3287	Hfr$_{11}$ M Sr	
WG4	W3703	Hfr L Tryp Sr	
H509a	W3479	F H Isol Sr	O25 K nm
	W3482	F H Isol Sr	O100 K+ H2

Markers without relation to the study have been omitted M = methionine L = leucine Tryp = tryptophane H = histidine, Isol = isoleucine and Sr = streptomycin resistant

W 3287 is a ...
selection (re...
resistance w...
medium The...
till n... Th...
be...

He...

(10^{-5} or less expressed as the observed ratio of recombinant to input parental cells) Strains termed Hfr (*Cavalli* 1950, *Hayes* 1953) show a high frequency of recombination in crosses with F strains (10^{-1} to 10^{-3}) In most sexual crossing experiments carried out so far both parent strains have been auxotrophic mutants which were unable to synthesise one or more compounds necessary for growth, most often amino acids This procedure requires considerable handling of each parent strain to produce the necessary mutants To simplify the screening of a large number of strains the so-called SRP-technique (streptomycin-resistance-prototroph) was developed (*Lederberg* 1951) Recombinants are selected from test crosses between auxotrophic, streptomycin-resistant tester strains and various prototrophic, streptomycin-sensitive *i e* wild type strains Thus a large number of coli wild type strains could be screened both for fertility and for mating type

METHODS

The parent strains were grown in penassay broth Difco for 20 hours and 0 5 ml from each parent culture was inoculated into a fresh broth The mixed culture was incubated for another 20 hours, centrifuged and the pellet resuspended in 0 5 ml distilled water One drop of this suspension was spread onto minimal medium (see below) Controls were provided by plating the washed parent cultures onto the same medium as used for crosses The plates were read after 48 hours incubation at 37° C No further analysis of the recombinant colonies was carried out (*Lederberg* 1951)

Media For fluid medium penassay broth Difco was most often used In some cases autoclaved ox heart infusion
was employed No difference
TMS agar plates (*Lederberg* 1'
and galactose 1 per cent was

Strains All type strains f
were examined

O antigen test strains	137
K antigen test strains	80
H antigen test strains	43

Two of the O type strains were found to be streptomycin resistant (O126 and O127), four O type strains have previously been found not to belong to *E coli* but to the *Citrobacter* group (*Orskov* 1956) and finally O47 has been lost many years ago Of the K type strains 29 were identical with different O type strains Among the 43 H antigen type strains 23 were already represented among the O or K type strains two H type strains have been found to belong the *Citrobacter* group The reduced number of different type strains examined was therefore

O antigen test strains	130
K antigen test strains	51
H antigen test sttrains	18
Total	199

76 *E coli* strains belonging to the OK group O26 K60 O55 K59 and O111 K58 in many different H antigen combinations were further examined (Table 1)

72 *Klebsiella* type strains representing the same number of different capsule antigens were also included

The auxotrophic coli strains with known fertility used in the crosses can be found in Table 2 These last mentioned strains were all developed in Dept of Genetics University of Wisconsin Madison Wisconsin U S A

TABLF 3 (cont)

Strain no	Serotype antigens O	K	H	W3287 K12 Hfr	W3103 WC4 Hfr	W31?? O10? F+	W31 9 O100 F-	W160? K12 F-
O-type strains								
H509a	*100*		2	++++	+++	++		–
H511	*10?*		8	++++	+++	++		–
H519	*103*		12	++++	+++	++		–
H521a	*106*		33	+++	+++			–
H70o	*107*		27	+++	++			–
H708b	*109*		*10*	+++		+++		–
26w	114		32	++++	–	++		
28w	*116*		10	+++	+	++		–
30w	*117*		4	+++	+	–		–
31w	*118*			+++	++		–	–
43w	123		16		+++	++++		
Canioni	*125*	*70B*	19	+++		++	–	
178/54	*129*		11	++++	+++	+++	–	–
4866/33	*130*		9	++++	+++	+++		–
S239	*131*		*26*	+++	–	+		–
N87	*132*		*28*	++	+	++		–
4370 53	*134*		*35*	+++	++	+	–	–
col Pecs	*135*		–	+++	++	+++	–	–
K type strains not listed above								
Pus 342? 41	7	7L	4	++	+++	+++	–	
H67	23	*22L*		+++	+++	+		
Bi449/42	9a	*264*	–	+	+++	+		–
E56b	8	*27A*		+	+++	–	–	–
H36	9	324	19		++		–	
A198a	9	364	19		++	++		
A262a	9	384			++	–	–	–
A12b	6	54L		+++	+++			
N24c	9	554			++	–		
5017/53	86ab	*64B*	*36*	++	++		–	
2160 53	127ab	*65B*	4	++	++		–	–
H type strains not listed above								
Bi7575 41	8	*25B*	*9*	++	++	–	–	–
H330b	8		*20*	++	++			–
K42	45		23		+			–
K72	*51*		*25*		+++	+++	–	
K181	11		33	++	+			–

\+ — 1 2 colonies
\+ — 10
++ — 10 50
+++ — 50–200
++ — > 200

In the serotype f rmulas means ...

O
K
H

methods (*Lederberg* 1950) After having been converted to the F+ state an Hfr mutant W3703 was isolated by the authors using indirect selection against a histidine less indicator W3703 has O antigen 25, no detectable K antigen and is non motile (non flagellate therefore missing H antigen)

H509a is the type strain of *E coli* O antigen *100* The markers *H Isol* and *Sr* were introduced by conventional methods W3482 received its F factor from the K12 strain W1876 F+ (*Orskov & Orskov* 1960a) W3479 and W3482 have O antigen O100 a K antigen not yet numbered and H antigen 2

TABLE 3

Crosses between Auxotrophic Hfr F+ and F Strains and Prototrophic E coli Antigen Type Strains

Strain no	Serotype antigens O	K	H	W3287 K12 Hfr	W3703 WC4 Hfr	W3482 O100 F+	W3479 O100 F−	W100 F12 F
O type strains								
U4/41	*4*	*3(L)*	5	++	−	−		
Bi623/42	*11*	*10(L)*	*10*	++	+++			
F10018/41	*18*	*76B*	*14*	++++	+++	++		
K12a	*17*	*16L*	*18*	++	++			
F8188/41	*19*	−	7	−	++			
F47a	25	*19L*	12	++++	+++	+		
P6a	*32*		19	++++	+++			
H304	*34*		10	++	−			
F77a	*35*		10	++++	±	+		
H510c	*37*		10		+	+++		
F11621/41	*38*		26	++++	++++	++++		
H7	*39*		−	+++				
H316	*40*		4	++++	++++	++		
H710c	*41*		40	++++	++	++		
P11a	*42*		37	++++	+++	+	±	
U19/41	*51*		24	++	+	±		
U20/41	*52*		10	+++	++++	++++		
Bi7327/41	*53*		3	++++	++	++++	++	
Su3684/41	*56*		−	++++	++	−		
F8198/41	*57*		−	++	++	+++		
F8962/41	*58*		27	+++	±			++
F10524/41	*62*		30		+++			
K6b	*64*			+++	++	++		
K11a	*65*		−	+++	++			
P1a	*66*		25	+++	+++			
P9b	*69*		38	+++	++			
P10a	*71*		12	++++	+++			
P12a	*73*		31	+++	+++			
F3a	*74*		19	+++	++++	++		+
E5d	*76*		8	++++	++			
F71	*80*		26	+++	+++	+	+	
H19	*84*		21	++++	++	++++		
H35	*86*		25	++	+++	++	+	
H40	*87*		12	++++	++++			
H68	*89*		16	++++	+++	++		
H77	*90*			+++				
H307b	*91*		−	++				
H308a	*92*		33		+++			
H319	*96*		19	+++	++			
H504c	*99*		33	+++				

All crosses were carried out at least twice and as could be expected the number of recombinants produced in a given cross could vary to a large extent from time to time

In addition to the coli type strains further 76 coli strains belonging to the OK groups O 26 K 60 O 55 K 59 and O 111 K 58 in a variety of H antigen combinations were tested (Table 4) These strains were isolated from outbreaks and single cases of infantile diarrhoea and in some cases from healthy or diseased animals Eleven strains gave productive crosses with W 3287 of these nine could also be crossed with W 3482 but these crosses were less productive None were fertile with the F strains W 3479 and W 1607 It should be pointed out that two strains out of three belonging to O 55 K 59 H 2 and that two out of two belonging to O 55 K 59 H 11 and furthermore three out of three belonging to O 26 K 60 H 32 were fertile in crosses with W 3287 In order to show that there were no direct epidemiological connection between these fertile strains of the same serotype the place where the strains were originally isolated have been recorded in the table In order to test if some *Klebsiella* strains were fertile in crosses with W 3287 the 72 *Klebsiella* capsule type strains were examined Eight were found to be streptomycin resistant and could not be tested with the SRP technique In none of the remaining cases recombinants could be detected

DISCUSSION

It appears from the recorded experiments that about one third of two hundred serologically different *E coli* strains are fertile in crosses with either or both of two Hfr testers having the K 12 fertility factor This figure is considerably higher than earlier reported figures (*Lederberg et al* 1952) As already mentioned Lederbergs screening of the fertility of wild type strains was carried out before the Hfr F and F mating system was adequately understood and an F strain was used as donor instead of the Hfr strain used here An F strain from the K 12 line was not included among the donor strains in this study but even the O 100 F strain W 3482 seems to detect more fertile coli wild type strains (18 per cent) than the K 12 F strain used in Lederbergs investigation One explanation for this discrepancy could be that the criteria for naming a strain E coli perhaps were less st[illegible]
the [illegible]
str[illegible]
patt[illegible] [illegible] pattern
with [illegible] deviations Another explanation might be the different origin and the different age as laboratory strains of the two series of strains The strains used by *Lederberg et al* were mostly freshly isolated strains from pathological conditions while most of the coli type strains have been kept in the laboratory for many years and only part of them

EXPERIMENTAL

199 different *E. coli* antigen type strains were crossed with the following strains of known fertility. Two Hfr strains W 3287 and W 3703 coming from the K 12 and the W G 4 line respectively, further one F+ strain W 3482 derived from the coli O 100 type strain. Finally crosses with two F⁻ strains W 1607 from the K 12 line and W 3479 derived from coli O 100 were carried out. The two last mentioned strains were included to see if any F⁻ strains were represented among the type strains. All positive crosses were carried out at least twice. The control plates on which the two parent strains were inoculated separatively showed no growth in the recorded cases.

TABLE 4

Crosses between Auxotrophic Hfr, F⁻ and F+ Strains and Prototrophic E. coli Strains Isolated from Infantile Diarrhoea

Strain no.	Serotypic antigens O K H	W 328 K 12 Hfr	W 3182 O100 F+	W 31 J O100 F	W 1607 K 12 F	Country [illegible]
C 24 55	26 60 32	+++	++			Switzerland
C116 55	26 60 32	+++	+++			Mexico
C [illegible] 56	26 60 32	+++	±			Wales
[illegible] 53 [illegible] 0	5 59 2	+++		+		Sweden
C572 54	55 59 2	++++				Germany
C 18 54	55 59 10	++++	+			
C 50 56	55 59 11	++++	++			Wales
C222 53	55 59 11	+++	+			France
C293 [illegible] 3	55 59 21	+++	++			
C238 56	55 59 25	+++	+			
C 87 [illegible] 3	111 58	+++	+			

+ = 1 2 [illegible]
+ = 10
++ = 10 50
+++ = 50–200
++++ = > 200

In the serotypic formulas [illegible] means not examined
[illegible] means antigen not present

The outcome of the crosses is shown in Table 3. It appears that 74 different strains were fertile in crosses with W 3287 or W 3703 i.e. 37 per cent. Sixtythree strains were fertile with W 3287 and sixtyfour with W 3703. Thirtyseven strains were fertile in crosses with the F strain W 3482. Generally there was good agreement between the fertile strains found in the three series of crosses, only 10 strains were fertile with W 3287 and not with W 3703 and 11 strains fertile with W 3703 and not with W 3287. All strains fertile with W 3482 gave productive crosses with either W 3287 or W 3703. Generally the number of recombinants was largest in the W 3287 crosses. Only a few examples of F+ strains i.e. strains giving recombinants in crosses with F⁻ strains were detected and the number of recombinants was low in these cases.

the O 26 K 60 or O 55 K 59 complex are fertile with the testers used The results seem to imply that the serotype is of some importance, because more cases were recorded where strains of the same serotype of independant origin were compatible

The simplest explanation would be that such fertile strains of the same serotype were derived from the same ancestral strain

With this explanation in consideration we cannot draw any conclusions from the recorded results as to a possible genetical or physiological connection between the antigenic composition of a certain serotype and its fertility

No large scale examination of the interfertility of the type strains that were found to be fertile in these studies have been carried out In a limited number of cases such fertile strains, after conversion to the F state could also be crossed with one another (*Ørskov & Ørskov*)

Two thirds of the examined strains were found to be sterile Future research will show if those strains are completely sterile or if they belong to other independant breeding groups Finally it should be kept in mind that the sterility detected might disappear when a different crossing technique was employed

SUMMARY

A screening for fertility of 199 *E coli* antigenic type strains (O, K and H antigens), using a number of testers of known fertility showed that about 35 per cent were fertile With few exceptions the strains were found in the F state

No definite correlation between fertility and single antigens could be found

A similar screening of a number of coli strains having relations to types found in infantile diarrhoea was performed In a number of cases there seemed to be some correlation between the serotype and the fertility

This fact is considered attributable, not to a connection of antigenic structure with fertility but to a common origin of the strains

Finally all *Klebsiella* capsule type strains were examined none were found to be fertile

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are from pathological conditions It is well known that the O and K antigens of freshly isolated strains are betlter developed than in old laboratory strains and further several investigators (*Kauffmann* 1944, *Vahlne* 1945) have shown that the frequency of O-inagglutinable strains, indicating strains with well developed K antigens, is greater among strains from pathological conditions than among strains isolated from normal faeces It is therefore probable that the strains examined by *Lederberg* had better developed K and O antigens, and further that the readiness with which they mutated to R forms was less than that of the old laboratory strains used in this study No body has yet reported if fertility in *E coli* is influenced by qualitative and quantitative differences of the O and K antigens, but it may be that strains with poor development of these antigens are more fertile In this connection it could also be pointed out that none of the 72 *Klebsiella* strains, which all have very large capsules were found to give productive crosses with the Hfr and F⁺ strains used here In another investigation (*Ørskov, Ørskov & Kauffmann* 1961) it was found that more than 50 per cent of randomly selected *Salmonella* strains representing all *Salmonella* O groups were fertile with the "K 12" Hfr strain used in this study, it is known that K antigens if present, are only poorly developed in *Salmonella* strains

Many of the O antigens of the coli type strains are interrelated, but only in very few cases have these relationships been definitely elucidated i e the coli antigenic scheme is not so explicitly developed as to give the highly differentiated tabulation of the different O antigen fractions which is characteristic of the Kauffmann-White scheme for the Salmonella group What does exist is a listing of the O antigen agglutination titres from mutual agglutinations of O test strains in O antigen typing sera (*Ewing* 1956) When such a list is compared with the result of the compatibility examination recorded in this paper, it is difficult to find any relationship between special O antigens or O antigen fractions and fertility

The K antigens of the examined strains are to a great extent not numbered yet, but from unpublished findings it is known that a large fraction of the unnumbered K antigens from the O series represent new and different K antigens most of them probably B antigens It is therefore also difficult to find any relationship between compatibility with the testers used and the specificity of the K antigens When we finally turn to the H antigens it is not possible to find any correlation between compatibility and the different H antigens One exception is H 10 which is found in 9 out of 130 strains in the O series 7 of these are fertile with one or more of the male testers

The outcome of the crosses involving more identical strains of the same serotype seem to tell more of the role of antigenic components in compatibility studies It appears that neither O nor K antigens alone can determine the compatibility, because only some of the strains with

THE FERTILITY OF SALMONELLA STRAINS DETERMINED IN MATING EXPERIMENTS WITH ESCHERICHIA STRAINS

By

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Received 11 x 60

In an earlier series of mating experiments a number of *Escherichia coli* serotypes were tested for fertility in crosses with a number of known *E coli* testers (*F Ørskov & I Ørskov* 1960) Stimulated by the reports of *Baron et al* (1958 1959) concerning the first successful *Salmonella* × *Escherichia* crosses we have attempted to screen a number of *Salmonella* serotypes for fertility with a technique similar to that used in the above mentioned *E coli* × *E coli* crosses

The present short paper gives an account of the result of these mating experiments further details of investigations into the mechanism of the *Salmonella* × *Escherichia* crosses will be given in subsequent communications

METHODS

The parent strains were grown in autoclaved ox heart infusion broth for 20 hours at 37° C and 0 5 ml from each parent culture was then inoculated into a fresh broth bringing the total volume to about 7 ml The mixed culture was incubated for another 20 hours without aeration centrifuged and the pellet resuspended in 0 5 ml distilled water One drop of this suspension was spread on to minimal medium (see below) Controls were provided by plating the wa hed par nt

ot u te times At least one lactose positive recombinant from each positive cross was typed serologically (O and H antigens) and was examined for its reaction in the series of biochemical tests ordinarily used in this laborat tests for the fermentat
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(Lederberg 1950) supplemented with lactose 1 per cent

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TABLE 1 (cont)

Group	No	Species	Serotype	W 3287 K 12 Hfr	D 133 WG 4 Hfr
J	913	S berlin	17 d 1 5	—	—
	634	S *michigan*	17 l v 1 5	++	—
	471	S usumbura	18 d 1 7	++++	—
K	100	S cerro	18 $z_4 z_{23}$	+++	—
	624	S siegburg	6 14 18 $z_4 z_{23}$ –	—	—
	883	S ghana	21 b 1 6	++++	—
L	218	S minnesota	21 b e n x	++++	—
	737	S magwa	21 d e n x	—	—
	659	S dakar	28 a 1 6	++++	—
M	1025	S halle	28 c 1 7	–	—
	106	S tel aviv	28 y e n z_{15}	+	—
	639	S ezra	28 z 1 7	—	—
	129	S urbana	30 b e n x	++++	—
N	632	S godesberg	30 g m	—	—
	271	S donna	30 l v 1 5	—	—
O	161	S adelaide	35 f g –	—	—
	196	S monschau	35 m t –	—	—
P	163	S inverness	38 k 1 6	—	—
	1048	S perth	38 y e n x	+	—
Q	735	S wandsworth	39 b 1 2	—	—
	169	S champaign	39 k 1 5	++++	—
	231	S riogrande	40 b 1 5	—	—
R	1044	S bukavu	1 40 l z_{28} 1 5	++++	—
	813	S bulawayo	1 40 z 1 5	++++	—
S	237	S waycross	41 $z_4 z_{23}$ –	++++	—
	918	S offa	41 z_{38}	+++	—
T	804	S kampala	1 42 c z_6	++++	—
	247	S weslaco	42 z_{38} –	++	—
U	333	S milwaukee	43 f g	—	—
	548	S kingabwa	43 y 1 5	—	—
V	433	S niarembe	44 a l w	—	—
	688	S guinea	44 z_{10}	—	—
W	723	S deversoir	45 c e n x	++++	—
	798	S dugbe	45 d 1 6	—	—
X	701	S bergen	47 i e n z_{15}	++++	—
Y	772	S dahlem	48 k e n z_{15}	++	—
	694	S djakarta	48 $z_4 z_{24}$ –	++++	—
Z	842	S greenside	50 z e n x	++++	—
51	975	S treforest	51 z 1 6	+	—
52	1096	S utrecht	52 d 1 5	—	—
53	1170	S humber	53 $z_4 z_{24}$	—	—

+ = 1 2 recombinant colonies
+ = < 10 recombinant colonies
++ = 10–50 recombinant colonies
+++ = 50–200 recombinant colonies
++++ = > 200 recombinant colonies

EXPERIMENTAL

86 *Salmonella* serotypes were crossed with the following *Escherichia* strains of known fertility W 3287, an Hfr strain developed from K 12 (against a lactose-negative indicator) and D 133, an Hfr strain derived from the WG 4 line All positive crosses were carried out at least twice

Strains

86 *Salmonella* strains selected at random representing all *Salmonella* O groups Details of the strain number and serotype are shown in Table 1 The data concerning the auxotrophic *E coli* strains with known fertility used as donor strains can be seen in Table 2, these strains were developed in the laboratory of Dr J Lederberg

TABLE 1

Crosses between Auxotrophic Escherichia Hfr Strains and Prototrophic Salmonella Strains

Group	No	Species	Serotype	W 3287 K 12 Hfr	D 133 WC 4 Hfr
	1	S paratyphi A	1 2 12 a –	—	—
A	2	S paratyphi A var durazzo	2 12 a –	—	—
	1017	S kiel	1 2 12 g p	—	—
	856	S abortus equi	4 12 – e n x	—	—
	6	S paratyphi B	4 5 12 b 1 2	–	—
B	5	S java	4 5 12 b –	+++	—
	31	S schleissheim	4 12 27 b –	—	—
	209	S typhi murium	4 5 12 i 1 2	—	—
	32	S paratyphi C	6 7 Vi c 1 5	++++	—
	33	S paratyphi C	6 7 c 1 5	—	—
C 1	34	S cholerae suis	6 7 c 1 5	—	—
	631	S decatur	6 7 c 1 5	—	—
	46	S *montevideo*	6 7 *g m s* –	—	—
	1210	S bareilly	6 7 y 1 5	+++	—
C 2	54	S muenchen	6 8 d 1 2		—
	50	S newport	6 8 e h 1 2	—	
C 3	175	S virginia	(8) d		—
	98	S kentucky	(8) 20 i z_{6}	—	
C 4	1200	S bornum	6 (7) (14) z_{38} –	+	—
	71	S sendai	1 9 12 a 1 5	—	—
	187	S miami	1 9 12 a 1 5	++++	—
D 1	59	S typhi	9 12 Vi d		—
	57	S typhi	9 12 d	++	
	115	S typhi T 2 AS	9 12 d	+	—
	65	S dublin	1 9 12 g p		
D 2	627	S strasbourg	(9) 46 d 1 7	—	
	510	S fresno	(9) 46 z_{38}	—	
E 1	101	S uganda	3 10 l z_{13} 1 5	++++	—
E 2	390	S binza	3 15 y 1 5	++++	
E 3	133	S illinois	(3) (15) 34 z_{10} 1 5	++	—
E 4	87	S senftenberg	1 3 19 g s t	+	
	197	S chittagong	(1) 3 10 (19) b z_{35}	++++	
F	90	S aberdeen	11 i 1 2	++	
	102	S rubislaw	11 *r e n x*		
G 1	499	S friedenau	13 22 d 1 6	+	
	91	S poona	13 22 z 1 6		
G 2	144	S mississippi	1 13 23 b 1 5	++	
	216	S worthington	1 13 23 z l w	++	
	93	S carrau	6 14 24 y 1 7	++++	
H	94	S onderstepoort	(1) 6 14 25 e h 1 5		
	518	S boecker	6 14 l v 1 7	+	
	375	S brazil	16 a 1 5	+++	
I	95	S hvittingfoss	16 b e n x	++	—
	96	S gaminara	16 d 1 7	—	—
	97	S kirkee	17 b 1,2	++	—

TABLE I (Cont.)

Group	No	Species	Semmge	W 3287 K 12 Hfr	D 133 W G 4 Hfr
J	913	S berlin	17 d L5	—	—
	634	S michigan	17 Lv L5	— —	—
	471	S usumbura	13 d L7	— — —	—
K	100	S cerro	13 $z_4 z_{23}$	— — —	—
	624	S siegburg	6 14 18 $z_4 z_{23}$ –	—	—
	833	S ghana	27 b L6	— — — —	—
L	218	S minnesota	27 b e.n.x	— — — —	—
	737	S marwa	21 d e.n.x	—	—
	652	S dakar	28 a L6	— — — —	—
M	1095	S halle	28 c L7	—	—
	106	S tel aviv	28 y e.n.z_{15}	—	—
	629	S ezra	28 z 17	—	—
	122	S urbana	30 b e.n.x	— — — —	—
N	637	S godesberg	31 g m	—	—
	271	S donna	30 Lv L5	—	—
O	161	S adelaide	35 f g	—	—
	196	S monschaui	35 m t –	—	—
P	163	S inverness	38 k L6	—	—
	1048	S perth	33 y e.n.x	—	—
Q	735	S wandsworth	39 b L2	— — — —	—
	169	S champaign	39 k L5	—	—
	231	S riogrande	40 b L5	— — — —	—
R	1044	S bukavu	L40 Lz_{28} L5	— — — —	—
	813	S bulawayo	L40 z 15	— — — —	—
S	237	S waycross	41 $z_4 z_{23}$ –	— — —	—
	918	S offa	41 z_{38} –	— — — —	—
T	804	S kampala	1 42 c z_6	— —	—
	247	S weslaco	42 z_{38} –	—	—
U	333	S milwaukee	43 f g –	—	—
	548	S kingabwa	43 y 15		
V	433	S niarembe	44 a 1 w	—	
	688	S guinea	44 z_{10} –	—	
W	723	S deversoir	4 c e.n.x	— — — —	
	709	S dugbe	45 d 16	—	
X	701	S bergen	47 i e.n.z	— — — —	
Y	772	S dahlem	48 k e.n.z	—	
	694	S djakarta	48 $z_4 z_{23}$ –	— — — —	
Z	842	S greenside	50 z e.n.x	— — — —	
51	975	S treforest	1 z 16	—	
52	1096	S utrecht	52 d 1		
53	1170	S humber	53 $z_4 z_{23}$ –		
			$z_4 z_{23}$ –	— — — —	

± = 1–2 recombinant colonies
\+ = < 10 recombinant colonies
\++ = 10–50 recombinant colonies
\+++ = 50–200 recombinant colonies
\++++ = > 200 recombinant colonies

EXPERIMENTAL

86 *Salmonella* serotypes were crossed with the [illegible] strains of known fertility W 3287, an Hfr strain [illegible] (against a lactose negative indicator) and D 133 an Hfr strain [illegible] from the W G 4 line All positive crosses were carried out [illegible]

Strains

86 *Salmonella* strains selected at random representing all *Salmonella* O groups Details of the strain number and serotype are shown in Table 1 The data concerning the auxotrophic *E coli* strains with known fertility used as donor strains can be seen in Table 2 *these strains were developed in the laboratory of Dr J Lederberg*

TABLE 1

Crosses between Auxotrophic Escherichia Hfr Strains and Prototrophic Salmonella Strains

Group	No	Species	Serotype	W 3287 K 12 Hfr	D 133 WC 4 Hfr
	1	S paratyphi A	1 2 12 a	—	—
A	2	S paratyphi A var durazzo	2 12 a	—	—
	1017	S kiel	1 2 12 g p	—	—
	856	S abortus equi	4 12 e n x	—	—
	6	S paratyphi B	4 5 12 b 1 2	—	—
B	5	S java	4 5 12 b	+++	—
	31	*S schleissheim*	*4 12 27 b*	—	—
	209	S typhimurium	4 5 12 i 1 2	—	—
	32	S paratyphi C	6 7 Vi c 1 5	++++	—
	33	S paratyphi C	6 7 c 1 5	—	—
C 1	34	S cholerae suis	6 7 c 1 5	—	—
	631	S decatur	6 7 c 1 5	—	—
	46	S montevideo	6 7 g m s	—	—
	1210	S bareilly	6 7 y 1 5	+++	—
C 2	54	S muenchen	6 8 d 1 2		—
	50	S newport	6 8 e h 1 2	—	—
C 3	175	S virginia	(8) d	—	—
	98	S kentucky	(8) 20 i z_6		—
C 4	1200	S bornum	6 (7) (14) z_{38}	+	—
	71	S sendai	1 9 12 a 1 5	—	—
	187	S miami	1 9 12 a 1 5	++++	—
D 1	59	S typhi	9 12 Vi d	—	—
	57	S typhi	9 12 d	++	—
	115	S typhi T 2 AS	9 12 1	±	—
	65	S dublin	1 9 12 g p		—
D 2	697	S strasbourg	(9) 46 d 1 7		
	510	S fresno	(9) 46 z_{38}	—	—
E 1	101	*S uganda*	*3 10 l z_{13} 1 5*	++++	—
E 2	390	*S binza*	*3 15 y 1 5*	++++	—
E 3	133	S illinois	(3) (15) 34 z_{10} 1 5	++	—
E 4	87	S senftenberg	1 3 1J g s t	+	—
	197	S chittagong	(1) 3 10 (19) b z	++++	—
F	90	S aberdeen	11 i 1 2	++	
	102	S rubislaw	11 r e n x	—	—
G 1	499	S friedenau	13 22 d 1 6	+	—
	91	S poona	13 22 z 1 6		
G 2	144	S mississippi	1 13 23 b 1 5	++	
	216	*S worthington*	*1 13 23 z 1 w*	++	
	93	S carrau	6 14 24 y 1 7	++++	
H	94	S onderstepoort	(1) 6 14 25 e h 1 5		
	518	S boecker	6 14 l v 1 7	+	
	375	S brazil	16 a 1 5	+++	
I	95	S hvittingfoss	16 b e n x	++	—
	96	S gaminara	16 d 1 7		—
	97	S kirkee	17 b 1 2	++	—

strain to the F+ state, together with the *demonstration of a simultaneously* acquired f+ antigen (*I Ørskov & F Ørskov* 1960 a) Except for three crosses, namely those involving no 471 *S usumbura*, no 87 *S senftenberg* and no 144 *S mississippi*, all the recombinants examined behaved exactly like the *Salmonella* parents both biochemically and serologically, except for lactose fermentation In contrast to the parent cultures the recombinants from the three exceptions did not ferment maltose

The recombination phenomenon described here thus seems to belong to the same category as the F² recombination of *Adelberg & Burns* (1959 1960), and the F duction of *Jacob et al* (1960)

In the light of the above mentioned results, the seemingly astonishing finding that 52 per cent of the *Salmonella* strains and only 31 per cent of the *E coli* strains (*F Ørskov & I Ørskov* 1960) were fertile with the same male *E coli* tester does not call for further comment, as the two figures are not comparable

TABLE 3

Crosses between E coli W 3287 and Smooth and Rough Forms of a Number of Salmonella Strains

Strain No		Species	W 3287 crossed with	
S	R		S	R
1	49	S paratyphi A	—	—
6	140	S paratyphi B	—	—
31	116	S schleissheim	—	—
33	117	S paratyphi C	—	—
34	147	S cholerae suis	—	—
206	225	S isangi	—	—
54	152	S *muenchen*	—	—
913	343	S berlin	—	—
163	65	S champaign	—	—
247	68	S weslaco	—	—

S — smooth R — rough

Reverting to the results in Table 1, it will be seen that the number of fertile *Salmonella strains seems to be* higher among the higher O groups If O groups A through D are taken together in one group and the remaining O groups in another group, we will find that 26 per cent of the first and 64 per cent of the second are fertile, in other words, it would appear that the first O groups are less fertile than those with higher numbers If the [illegible] number of strains it might be [illegible] between the low and the high [illegible] eme, e g the decrease in complexity of the sugar components in the cell walls (O antigens) which is found when going from the low to the high O antigen numbers (*Kauffmann et al* 1960) In a paper by *Lüderitz et al* (1960) it was reported that R (rough) strains had fewer and

The control plates on which the parent strains were inoculated separately showed no growth

TABLE 2
Auxotrophic E coli Strains Used

Source	Strain designation	Markers	Serotype
K 12	W 3287	Hfr$_{13}$M Sr Lac$^+$	0 K? H48
WG 4	D 133	Hfr L Tryp Sr Lac$^+$	025 K nm

Markers without relation to the study have been omitted
M = methionine L = leucine Tryp = tryptophane, Sr = streptomycin resistant and Lac$^+$ and Lac means lactose fermenting or non fermenting W 3287 is an Hfr strain isolated from K12F$^+$M Lac$^+$ by J Lederberg using indirect selection (replica plating UV survivors against a Lac indicator) Streptomycin resistance was introduced by selection of a spontaneous mutant on streptomycin medium The K 12 strains are characterized as rough, no O antigen has been found up to now The presence of an ordinary K-antigen is doubtful The H antigen is numbered H 48 (*I Ørskov & F Ørskov* 1960 b) WG4 was isolated in 1950 from a urine culture submitted to the Wisconsin Public Health Laboratory The markers L Tryp and Sr were introduced by conventional methods (*Lederberg* 1950) After having been converted to the F$^+$ state, an Hfr mutant W3703 was isolated by the authors using indirect selection against a histidine less indicator (*I Ørskov & F Ørskov* 1960 a) From W3703 which is Lac , a spontaneous Lac$^+$ mutant D133, was selected on lactose medium D133 has O antigen 25 no detectable K antigen and is nonmotile (non flagellate therefore lacking H antigen)

The outcome of the crosses is recorded in Table 1 It will be seen that out of 86 *Salmonella* strains examined, 45 or 52 per cent, would give reproductive crosses with W 3287 When crossed with D 133 all strains were non-fertile D 133 is a lactose-positive mutant of W 3703, which was used alongside W 3287 in the fertility screening of *E coli* strains reported previously In those experiments the fertility detected with W 3703 and with W 3287 to a large extent ran parallel In order to see whether D 133 could give reproductive crosses when mated with *E coli* strains, using lactose fermentation and prototrophy as selective markers, we looked for a lactose negative, fertile *E coli* strain

The test strain for *E coli* O 130 is fertile in SRP cross with W 3703 (*F Ørskov & I Ørskov* 1960) and ferments lactose mutatively only after 5 6 days In contrast to the *Salmonella* strains, it gave a rich yield of recombinants when crossed with D 133 on EM lactose This fact, together with some other findings, suggested strongly that a fundamental difference exists between the type of cross reported here and the *E coli* × *E coli* crosses reported previously A more detailed account of investigations into the special character of the reported crosses will be published elsewhere, it will suffice to say that in their instability of the Lac marker the recombinants resemble the diploids of *Lederberg* (1949) and furthermore that the recombinants have acquired the F$^+$ factor together with the Lac marker This latter fact was proved by the demonstration of the ability to convert a K 12 F

FROM THE TREPONEMATOSIS DEPARTMENT
STATENS SERUMINSTITUT COPENHAGEN

THE STABILITY OF ANTI LIPOIDAL ANTIBODIES TO HEAT INACTIVATION AT 56° C

By

HENNING SCHMIDT

Received 14 xi 60

Most of the techniques in syphilis serology employ inactivation of sera consisting of heating the serum prior to titration of the antibody content Most authors use heating at 56° C on a water bath for 30 minutes but other schedules for inactivation have been used for example 60° C for five minutes

The somewhat longer inactivation time at 56° C has the advantage that a minor contravention of the period for heat treatment of the serum is of no decisive importance The risk of coagulation of serum is rather slight at temperatures about 56° C At the State Serum Institute inactivation of sera at 56° C for 30 minutes prior to the serological investigations for syphilis has been the standard procedure since *Boas* (1910) introduced the Wassermann technique for complement fixation tests

Sachs & Altmann (1908) reported a case where serum from a syphilitic patient reacted in the active state but was non reactive after inactivation *Boas* (1910) examined this phenomenon in greater detail and observed that a serum in the active state usually showed a higher titre than found with the same serum after inactivation In the majority of syphilitic sera the antibody content was found to decrease and in 21 sera from non syphilitic patients which were reactive when examined in the active condition the reactivity disappeared completely as a result of heating

Kvittingen (1952) examined the influence of serum inactivation on biological false positive sero reactions Using sera from non-syphilitic leprosy patients he compared the results in the Meinicke clarification test in which active serum is used with the results obtained in the VDRL test For the VDRL test the sera were inactivated at 56° C for 30 minutes or alternatively at 60° C for four minutes or at 65° C for five minutes Kvittingen found that raising the temperature of inactivation resulted in a reduction of the number of reactive sera He found that when the sera from leprosy patients with positive sero reactions for syphilis were merely stored at room temperature their serological reactivity decreased

simpler sugar components than the corresponding S (smooth) forms Table 3 shows the outcome of crosses between *Salmonella* R forms derived from some of the non-fertile S forms listed in Table 1 The crosses were carried out simultaneously with S and R forms As it will be seen, the 10 strains examined were as non-fertile in the R state as in the S state

SUMMARY

A short report is given concerning screening of the fertility of 86 *Salmonella* strains representing all *Salmonella* O groups in crosses with two different *E coli* Hfr strains Fifty two per cent of the *Salmonella* strains are fertile with one of them, and none with the other Twenty six per cent of *Salmonella* strains belonging to O groups A–D and 64 per cent of higher O groups were fertile The *Salmonella*–*Escherichia* recombinants were lactose-positive and serologically identical with the *Salmonella* parents In this way lactose-positive *Salmonella* hybrids were produced

It is suggested that the fertility detected is determined by the episomal transfer of the markers F^+ and Lac^+

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*It is suggested that the fertility detected is determined by the epi*somal transfer of the markers F and Lac⁺

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(a) CWRM

	inactive sera +	inactive sera —
active sera +	10	68
active sera —	1	80

(b) Cardchol

	inactive sera +	inactive sera —
active sera +	50	60
active sera —	1	48

Fig 2

The relationship between the reactivity of active and inactive sera in CWRM and Cardchol for TPI non reactive sera

There were 159 TPI non reactive sera in the material, and of these, 78 sera were reactive in CWRM in their active condition, but only 11 sera were reactive in CWRM after inactivation (Figure2 a) One serum which was non reactive in the active form, became reactive in CWRM after inactivation Of the 78 sera which were reactive in CWRM in their active form, there were only 10 sera, 13 per cent, which remained reactive after inactivation, which implies that 87 per cent of sera reactive in their active condition, lost their reactivity on inactivation

Of the 159 TPI non-reactive sera, 110 were reactive with Cardchol in their active condition, while only 51 were reactive with Cardchol after inactivation (Fig 2 b) Of these 51, however, one serum was non-reactive in its active condition, and became reactive after inactivation Thus of 110 sera which were reactive in their active condition, 60, or 55 per cent, lost their reactivity on inactivation

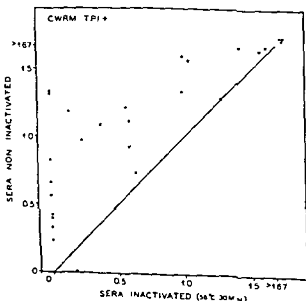


Fig 3

Prompted by Kvittingen's observations, it was felt desirable to examine the influence of inactivation on the antilipoidal antibodies demonstrated in complement fixation tests Furthermore, as a considerable material of sera from leprosy patients was received for serological examinations for syphilis, and as many of these sera occurred in such amounts as to permit an examination of the serum in both active and inactive condition, these sera form the basis for the present study

MATERIAL AND METHODS

309 sera from leprosy patients were received from *Dr C W Chacko* Institute of Venereology, Madras India for examination by various lipoidal antigens (*Schmidt* 1961 b), and 189 of these contained sufficient material to permit a determination of the antibody content in both active and inactive serum

All sera were examined concurrently in the active and inactivated form by means of two complement fixation tests viz Wassermann Mørch's complement fixation test using cardiolipin antigen CWRM (*Schmidt* 1951, 1952 *Schmidt & Lundback* 1954), as well as with a lecithin free cardiolipin antigen, Cardchol in a complement fixation text according to almost the same technique as CWRM (*Schmidt* 1955 a b, 1959 a b 1961 b) In addition the sera were examined in the TPI test (*Nelson & Mayer* 1949 *Nielsen* 1957)

RESULTS

The material was divided into two groups according to the result of the TPI test, *viz* sera which were TPI-reactive and sera which were TPI-non-reactive The reactivity of CWRM and Cardchol was then compared for active and inactive serum, within both the TPI groups

(a) CWRM

	inactive sera +	inactive sera —
active sera +	18	9
active sera —	1	2

(b) Cardchol

	inactive sera +	inactive sera —
active sera +	22	5
active sera —	0	3

Fig 1
The relationship between the reactivity of active and inactive sera in CWRM *and Cardchol for TPI reactive sera*

A total of 30 out of the 189 sera were reactive in TPI Figure 1(a) shows that 18 sera examined in CWRM were reactive in both active and inactive condition Nine sera which were reactive in CWRM in the active condition, were non-reactive after inactivation One serum was reactive after inactivation, but non-reactive in the active condition, and finally, two sera were non reactive both before and after inactivation Nine out of 27 TPI-reactive sera, 33 per cent, lost their reactivity in CWRM after heating the serum to 56° C for 30 minutes

Figure 1(b) shows that of the 30 TPI-reactive sera, 22 were reactive with Cardchol, in both active and inactive condition Five sera reacted in the active condition, but were non reactive after inactivation Finally, three sera were non reactive with Cardchol, in both the active and inactive condition Five out of 27 TPI reactive sera 18 per cent, lost their reactivity on inactivation

sity of inactivation of sera prior to examination with Kahn antigen, by comparing the reactivity of both active and inactive sera, examined with Kahn's antigen In the active condition, the sera showed no or only insignificant reactivity, in spite of the fact that the same sera were clearly reactive after inactivation (unpublished observation)

To judge from the present material heat inactivation does not seem to have a constant effect on sera Sera which are reactive in TPI in addition to their reactivity with lipoid antigens, are more stable, i e on the average their titre decreases less than that of sera which are lipoid antigen reactive, but TPI non reactive In the present as in previous series, a reactive TPI test is considered as expressing a treponematosis at one time or other in the life of the patient in question Similarly, a non reactive TPI is regarded as indicating that the patient did not suffer from any treponematosis at the time of the investigation Within the latter category, the reservation must be made that recently acquired treponemal infections do not cause the formation of demonstrable amounts of immobilizing antibody until they have persisted for some time

Neurath, Voll [illegible] 947) examined the suscepti [illegible] false positive sera to tem [illegible] enty minutes Their investigations revealed that the titres of both types of sera de*creased with increasing temperature, the temperature at which com*plete inactivation occurred being lower for biologic false positive sera than for syphilitic ones of comparable initial serologic activity

Kuttingen (1952), as mentioned, was able to show that presumably non treponemal antibodies, demonstrated by VDRL were reduced merely by storage at room temperature, and that inactivation at varying temperatures weakened the antilipoidal antibodies to a degree proportional to the temperature of inactivation

The poor stability of the non treponemal antilipoidal antibodies might perhaps also explain the very varied reports of the frequency of activity of different lipoidal antigens in leprosy as the duration of the shipment by air m [illegible] ne content of demonstrable [illegible] might be mentioned that *Fr* [illegible] total of 318 patients with lepromatous leprosy found a frequency of reactivity with lipoidal antigens of approximately 3–4 per cent This very low reactivity percentage may depend on sera being freeze dried before [illegible] *test lab* [illegible]

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Those antibodies demonstrated with lecithin free cardiolipin antigen appear to be considerably more stable towards the effect of heat than those antibodies which react with cardiolipin antigen of normal com-

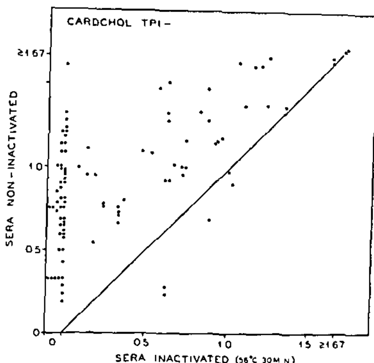


Fig. 6

tendency is not so pronounced as with CWRM in the case of the TPI-non-reactive sera. The fall in titre for the sera which retain some reactivity after inactivation seems to lie on the average around 0.5 log_{10} val, but quite a considerable proportion of the sera which in their active condition have a titre of 0.7 log_{10} val and above, lose their reactivity completely on inactivation.

DISCUSSION

Originally, it was *Wassermann*'s intention that the inactivation of sera prior to examination for complement-fixing antibodies should destroy the unknown amount of complement which is present in all sera. Following the inactivation, there was thus no other complement than that added in the form of guinea-pig serum. If, on the strength of its haemolytic property, the complement normally present in human serum should play a role for the result of the sero-reaction, it must be expected that active sera would have a lower titre than sera after inactivation. Exceptionally, this can be the case for certain sera, but as a general rule it can be considered that there is a fall in titre on heating the serum.

The contrary is, however, the case in the Kahn reaction. *Kahn* (1928) recommends that the sera be heated to 56° C before examination with Kahn antigen, and reinactivated by heating to 56° C for 10 minutes in subsequent examinations. The inactivation is supposed to enhance the flocculation in the sero-reaction. It was possible to confirm the neces-

sity of inactivation of sera prior to examination with Kahn antigen, by comparing the reactivity of both active and inactive sera, examined with Kahn's antigen In the active condition the sera showed no or only insignificant reactivity in spite of the fact that the same sera were clearly reactive after inactivation (unpublished observation)

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The poor stability of the non treponemal antilipoidal antibodies might perhaps also explain the very varied reports of the frequency of activity of different lipoidal antigens in leprosy, as the duration of the shipment by air must necessarily influence the content of demonstrable antilipoidal antibody In this connexion it might be mentioned that *Fromm Fuhner, Ruge & Guinto* (1959), in a total of 318 patients with lepromatous leprosy found a frequency of reactivity with lipoidal antigens of approximately 3–4 per cent This very low reactivity percentage may depend on sera being freeze *dried before dispatch to the* test laboratory and then reconstituted and inactivated It is poss[illegible] that the process [illegible] on the antil[illegible] tion

Those antibodies demonstrated with lecithin free cardiolipin antigen appear to be considerably more stable towards the effect of heat than those antibodies which react with cardiolipin antigen of normal com

position The fact that the Cardchol reaction is so much more non specific than CWRM may possibly be explained to some extent by this

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MECHANISM OF EXPERIMENTAL TUMORIGENESIS

7. *Ultrastructural Changes in Mouse Epidermis Caused by Glyceryl Stearate-Type Tumor Enhancers in Acetone and in Water*

By

KAI SETÄLÄ, LEO STJERNVALL, MAURI NYHOLM, LAURI MERENMIES, EINO ERKKI NISKANEN and YRJÖ AHO

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It has been shown that certain laboratory-synthesized glyceryl stearates of the types of Span 60 and Tween 60 enhance development of local tumors in mouse skin (1). In acetone solution the same tumor response was obtainable with about 1/90th of the dosage necessary in aqueous solution. Such results are hardly surprising, as the mammalian epidermis is not a water-absorbing organ.

It has also been shown that, in the epidermis of mice of a tumor-resistant strain, the hyperplastic response after exposure to dipole-type tumor enhancer is essentially different from that which follows exposure to carcinogens. In some respects the two types of response may be regarded as opposites (reviewed in (2)). The difference also manifests itself in electron microscopy: dipole-type tumor enhancers maintain the ultrastructure of nucleated epidermal cells and mitochondria (3), whereas carcinogenic hydrocarbons cause development of new cell types, immaturity and disturbances in keratinization and, as a rule, mitochondrial changes and accumulation of globular inclusion bodies within mitochondria (2, 4).

The aim of the present electron-microscopic study was to investigate whether or not exposure of the mouse back to laboratory-synthesized, pure tumor enhancers of the type of glyceryl stearates results in a maintenance of the ultrastructure of the epidermal cells similar to that following treatment with technical and laboratory-synthesized agents of the type of Tween 60 (3). The agents were administered both in aqueous and in acetone solutions.

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MATERIALS AND METHODS

The details of the general experimental procedure and those of the light microscopic histoquantitative and electron microscopic techniques have been given in previous papers (e g 1 5) All experiments were performed on the rand mly bred mice of this laboratory s tumor resistant strain

The glyceryl stearates were synthesized in this laboratory and administered continuously 6 times a week Sundays excluded They comprised the following agents glyceryl 1 stearate (compound I) and glyceryl 1 2 distearate (II) both of which are Span 60 type agents as well as glyceryl 1 stearate 2 3 *bis* polyoxyethylene ether (III) and glyceryl 1 2 distearate 3 polyoxyethylene ether (IV) which are agents of the type of Tween 60 Compounds III and IV were made from compounds I and II by adding on an average 15 and 30 units of ethylene oxide respectively This was done to obtain about the same hydrophile lipophile balance value as that of Tween 60 Owing to the solubility properties compounds I and II were used only as 0 0066 *M* in reagent grade acetone whereas compounds III and IV were applied both as 0 0066 *M* in acetone and as 0 18 *M* in aqueous solution The latter strength is the same as that of the technical and laboratory synthesized Tween 60 in previous experiments (*e g* 3) The aqueous solutions were administered onto the skin by brush painting the acetone solutions by dropping The theoretical single dose at the cutaneous surface was calculated at in the acetone series 0 13 mg of I 0 16 mg of II 0 33 mg of III and 0 60 mg of IV and the water series 30 mg of III and 60 mg of IV Thus the dose given in water was about 90 (91) to 100 times the corresponding dose given in acetone

In experiments with the two stage technique initiation was made with 9 10 dimethyl 1 2 benzanthracene (DMBA) from L Light & Co Colnbrook England the dose being 320 γ in colorless non fluorescent light paraffin The solution was carefully dropped from a glass pipette onto the center of the treatment area on the back and then spread with a glass rod Wetting of the surrounding fur was avoided Posttreatment with glyceryl stearates was commenced after an interval of 30 days The hair within the treatment area was cut at one week intervals

In experiments without initiation skin biopsies were taken from 5 animals in each series on the 2d 6th 10th 16th 30th and 60th days from commencement of experimental series In experiments with the two sta
followed but the first biopsy was taken on t
Thus each series contained 30 animals which

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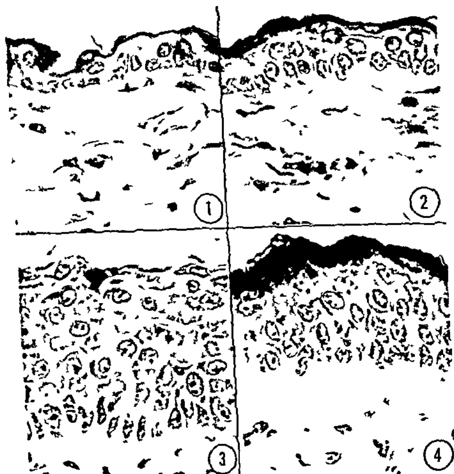
RESULTS

Experiment A Light Microscopy

Series without initiation —Exposure of the skin to compound I produced only insignificant changes in the interfollicular epidermis (IFE) No intercellular spaces developed Later on the 16th and 30th days (Fig 1), the IFE was in places somewhat thicker than normal

Exposure to compound II brought about a significantly stronger response (Fig 2)

The alterations in the IFE caused by acetone solutions of compounds III and IV (Fig 4) differed essentially from those evoked by compounds I and II a comparatively high degree IFE hyperplasia developed rapidly being most marked in the 60th day biopsies Series treated with compound IV had a stronger hyperplastic response than those treated with compound III in degree it was about the same as that previously seen in mice treated with Tween 60 (6 8) Aqueous



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F g 1 Mou e N 9897 0 0066 *M* acet ne soluti n of glyceryl 1 stearate (I) 30th day IFI nly sl ghtl thicker than normal Cells nearly n rmal in size shape and num ber N intercellular spaces cell boundar es and stinct Ha r f llicular cycle telogen

F g 2 Mouse No 9930 0 0066 *M* acetone solution of glyceryl 1,2-d stearate (II) 30th day IFE sh ws a m lerate hyperplastic response Stratum corneum thicker and m re c arse than normal Ha r f ll cular cycle anagen

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F g 4 M u e N 9988 0 0066 *M* acet ne s lution of glyceryl 1,2-d stearate 3 poly xyethylene ether (IV 30th day rather high degree but somewhat uneve IFE hyperplas a Nuclei larger than n rmally Note the degree of ep dermal response th ugh the I e of th is compound in *acet ne was nly 1 100th* of that in water (Fig 3) Hair foll c lar cycle telogen

solutions of compounds III and IV also caused a high-degree hyperplasia of the IFE. Intercellular spaces developed, and intercellular bridges were distinguishable between basal cells (Fig 3). Changes in the IFE were *more uniform than in the acetone series. In water, too,* compound IV appeared to be more effective than compound III.

None of the four compounds caused cellular or nuclear atypia. The pilo-sebaceous apparatus was intact. The epidermal alterations were accompanied by inflammation of the dermis.

Series with DMBA initiation.—Exposure of the skin to acetone solutions of compounds I and II gave rise to an increase in the total cell count. The response of the IFE to compound II was stronger than that to compound I just as in the series without initiation.

The effect upon the IFE of compounds III and IV both in acetone and in water was significantly stronger than that of compounds I and II, compound IV being the most effective. Characteristic of the alterations in the IFE in the DMBA-initiated series was development of and increase in compactly-built basal-type cells. This has been previously shown to occur in the IFE as a result of exposure of mouse skin to technical and laboratory-synthesized Tween 60 and Tween 40 after local (6, 9) and remote (10) carcinogen initiation.

The hair follicular cycle has no effect on the number of cells and cell layers or the cellular composition of the IFE as also appears in our earlier light-microscopic studies with Span 60 and Tween 60 (6, 7, 10).

To sum up: Treatment of the mouse back with glyceryl-l-stearate (I), both alone and after DMBA initiation, caused alterations in the IFE similar to those obtained with Span 60 in related experiments. Similarly, treatment with glyceryl-1-stearate-2,3-*bis*-polyoxyethylene ether (III) and glyceryl-1,2 distearate-3 polyoxyethylene ether (IV) resulted in changes identical with those in corresponding Tween *60* series. Glyceryl-1,2-distearate (II), which contains no ethylene oxide, affected the IFE in a manner largely resembling that of Tween 60-type tumor enhancers. When compounds II, III and IV were dissolved in acetone, about 1/90th to 1/100th of the dosage necessary in water sufficed to bring about a nearly high degree IFE hyperplasia.

Figures 5 to 12 are electron micrographs. Labels on the micrographs indicate cellular components as follow: *bad* – basal attachment devices, *cb* – cell boundaries, *col* – collagen bundles, *d* – desmosomes, *dej* – dermoepidermal junction, *ib* – intercellular bridges, *is* – intercellular spaces, *L* – leukocytes, *m* – mitochondria, *n* – nuclei, *t* – tonofibrils and *tf* – tonofilaments.

Fig 5

Mouse No 9977. 0.0066 *M* acetone solution of glyceryl 1,2 distearate 3 polyoxyethylene ether (IV), 10 th day: an electron micrograph from the basalmost portion of medium degree IFE hyperplasia illustrating that exposure to compound IV that ... s about the typical "Tween 60 effect" ... m finely granulated. By (×) is indi... s as intracellular edema. Hair folli... en × 38 000


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Experiment B Histoquantitative Analysis

The results of histoquantitative analyses of the same specimens were in accord with the light-microscopic patterns As in previous experiments with Span 60 and Tween 60 (6–9), a direct correlation was found to exist between the tumor-enhancing efficacy of compounds II through IV (1), and their ability to provoke hyperplasia in the IFE

Experiment C Electron Microscopy

Series without initiation.—The general electron-microscopic appearance agreed with the light-microscopic pattern of the same material (Experiment A) In addition, electron microscopy revealed that the alterations in the IFE brought about by exposure of the skin to compounds I and II, and III and IV, were related to those previously found to be characteristic of Span 60 and of Tween 60, respectively (3) Therefore it suffices here to emphasize a few important characteristics only

Exposure of the skin to compound I gave rise to the same ultrastructural patterns in the IFE as similar treatment with technical and laboratory-synthesized Span 60

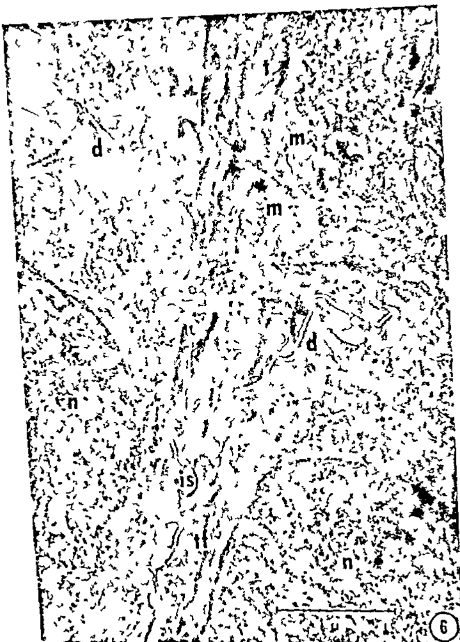
Compound II on the other hand brought about hyperplasia of the IFE

Compounds III and IV (Fig 5) both of which are Tween 60 type agents obtained by addition of ethylene oxide units to compounds I and II, caused changes similar to those previously seen after treatment with technical and laboratory-synthesized Tween 60 As in light microscopy, a somewhat varied response of the IFE was conspicuous in different places of the same treatment area after administration of compounds III and IV in acetone Apart from hyperplasia of the IFE, a variation in width of the intercellular spaces was characteristic of the acetone series, in places, no distinct intercellular spaces could be distinguished On the contrary, after treatment with these same compounds in aqueous solution, the development of distinct, wide intercellular spaces was evident throughout the treatment area just as in series treated with Tween 60 However, it must be borne in mind that the doses of compounds III and IV given in aqueous solutions were from 90 to 100 times higher than those administered in acetone Three ultrastructural patterns were characteristic of the effect of compounds III and IV both

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Fig 6

Mouse No 17351 0 18 *M* aqueous solution of glyceryl 1 stearate 2 3 *bis* polyoxyethylene ether (III) 30th day an electron micrograph from the middle layer of hyperplastic IFE The high degree preservation of ultrastructural patterns characteristic of the effect of Tween 60 type agents is evident *Note* particularly the normal *profile of the* mitochondria Typical of the effect of glyceryl stearates was ... in this figure intercellular spaces are almost ... follow each other Desmosomes are in places ... follicular cycle telogen × 38 700


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in acetone and in water: first, intercellular bridges were formed even between basal-type cells (Fig 5); secondly, the cytoplasm of nucleated cells was distinctly enlarged; and, thirdly, a particularly typical feature was the intercellular edema that appeared in the perinuclear halo in the form of vacuoles. In Figure 5, *v* indicates such a pattern obtained by treatment with compound III The cytoplasm of the large, edematous nucleated cells, even in relatively superficial layers of the hyperplastic IFE, maintained its granular, sol-like ultrastructure for a long time (Fig 6) The ultrastructure of the mitochondria, including their internal cristae, of the nucleated cells appeared normal (Figs 5 to 7) Further, the dermoepidermal junction showed profiles of the kind previously found both in normal IFE (5) and in mice treated with Tween 60 (3) Nor did the electron-microscopic appearance of the desmosomes deviate from normal (5) These patterns were also found in series treated with compound II, which does not contain ethylene oxide chains In the mice treated with compounds III and IV, the above patterns were unaffected by the duration of the exposure and by the solvent. acetone *vs* water

Series with DMBA initiation — As in the uninitiated series, light and electron microscopy showed that the changes of the IFE were in accord Further, the ultrastructural alterations in the present, initiated series were in principle similar to those previously obtained with agents of the types of Span 60 and Tween 60 after initiation (4)

Exposure of the skin to compound I evoked a weak hyperplastic response in the IFE related to that ensuing after the corresponding treatment with technical and laboratory-synthesized Span 60

In the early stages of the treatment period, compound II gave rise to relatively sparse alterations only Later a clear hyperplasia of the IFE was observed Figure 8 shows the relatively good preservation of various ultrastructural profiles In places, the cytoplasmic matrix showed more fibrillar and/or filamentous patterns than that of the basal cells of untreated IFE

In series posttreated with compounds III and IV, the ultrastructural appearance was indenpendent on the solvent acetone (Fig 9) and water (Fig 10) Characteristic of the changes in the IFE resulting from exposure to compounds III and IV after DMBA initiation was a

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Figs 7 8

Fig 7 Mouse No 9957 0 0066 *M* acetone solution of glyceryl 1 stearate 2 3 *bis*-polyoxyethylene ether (III) 30th day an electron micrograph from the middle or upper layers of hyperplastic IFE Also here is evident a high degree preservation of ultrastructural patterns (*cf* Fig 6 *the same compound in water) Hair follicular cycle* anagen × 51 600

Fig 8 Mouse No 9796 0 0066 *M* acetone solution of glyceryl 1 2 distearate (II) beginning 30 days after a single initial local application of 320 γ of DMBA 10th day an electron micrograph from the upper poles of two basal cells *The matrix of some* mitochondria (*arrows*) appear electron dense and the internal cristae are not visible [*cf* Fig 9 and electron micrographs in (4) Hair follicular cycle anagen × 45 300

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considerable variation in degree and quality in different places of the treatment area even in the same specimen Further electron microscopy revealed patterns resembling the effects both of DMBA (4) and of Tween 60 (3) sometimes even in the same cell Intercellular spaces were irregular in size and shape The intercellular bridges were often irregular and stretched The otherwise normal looking desmosomes were directed along the axis of the stretched bridges Generally the cytoplasm was finely granulated (Figs 11 and 12) and intracytoplasmic vacuoles and/or edema which were characteristic of the exposure of the skin to compounds III and IV in series without DMBA initiation were not seen Sometimes a paramembraneous crown of thorn like pattern was encountered (*cf* 4) Most of the mitochondria appeared to have retained their typical ultrastructure (Figs 11 and 12) However here and there alterations were evident (Figs 8 9 and 11) Sometimes these changed mitochondria were enlarged the internal cristae were irregular and intracristally there were one or more round electron dense inclusion bodies (Fig 9)—similar to those previously seen in initiated series (4)

The phase of the hair follicular cycle had no detectable effect on the ultrastructure of the hyperplastic alterations in IFE brought about by exposure to glyceryl stearate type tumor enhancers (*cf* 3)

To sum up Exposure of the mouse back to glyceryl 1 stearate 2 3 *bis* polyoxyethylene ether (III) and glyceryl 1 2 distearate 3 polyoxyethylene ether (IV) and to some extent to glyceryl 1 2 distearate (II) brought about ultrastructural patterns in the IFE that were closely related to those caused by Tween 60 type tumor enhancers—the "Tween 60 effect Thus the most prominent characteristic was a high degree maintenance of the ultrastructural profiles of the nucleated cells On the other hand exposure to these same compounds after preceding DMBA initiation caused changes that resembled those brought about with Tween 60 after similar DMBA initiation

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Figs 9 10

Fig 9 M se N 98 7 0 00 e oxyethylene ether 320 γ f DMBA 1 plastic IFE Interc regular Mitoch u ia arro s) contain electron-dense inclusion bodies Hair follicular cycle telogen × 30 00

Fig 10 Mo e No 17311 0 18 *M* aque us s luti n f glyceryl 1 2 xyethylene eth r IV 320 γ f D plastic IFE stretched a ti kiness d e f th l that in Fig 10 Hair follicular cycle anagen × 7,80 ugh the

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considerable variation in degree and quality in different places of the treatment area, even in the same specimen. Further, electron microscopy revealed patterns resembling the effects both of DMBA (4) and of Tween 60 (3), sometimes even in the same cell. Intercellular spaces were irregular in size and shape. The intercellular bridges were often irregular and stretched. The otherwise normal-looking desmosomes were directed along the axis of the stretched bridges. Generally the cytoplasm was finely granulated (*Figs 11 and 12*), and *intracytoplasmic* vacuoles and/or edema, which were characteristic of the exposure of the skin to compounds III and IV in series without DMBA initiation, were not seen. Sometimes a paramembranous crown of thorn-like pattern was encountered (*cf* 4). Most of the mitochondria appeared to have retained their typical ultrastructure (Figs 11 and 12). However, here and there alterations were evident (Figs 8, 9 and 11). Sometimes these changed mitochondria were enlarged, the internal cristae were irregular, and intracristally there were one or more round, electron-dense inclusion bodies (Fig 9)—similar to those previously seen in initiated series (4).

The phase of the hair follicular cycle had no detectable effect on the ultrastructure of the hyperplastic alterations in IFE brought about by exposure to glyceryl stearate-type tumor enhancers (*cf* 3).

To sum up: Exposure of the mouse back to glyceryl-1-stearate-2,3-bis-polyoxyethylene ether (III) and glyceryl-1,2 distearate-3-polyoxyethylene ether (IV), and, to some extent, to glyceryl-1,2 distearate (II) brought about ultrastructural patterns in the IFE that were closely related to those caused by Tween 60-type tumor enhancers—the "Tween 60 effect". Thus the most prominent characteristic was a high degree maintenance of the ultrastructural profiles of the nucleated cells. On the other hand, exposure to these same compounds after preceding DMBA initiation caused changes that resembled those brought about with Tween 60 after similar DMBA initiation.

Figs 9–10

Fig 9 M[illegible] [illegible] intercellular bridges somewhat irregular. Mitochondria (arrows) contain electron-dense inclusion bodies. Hair follicular cycle: telogen. × 30 700

Fig 10 Mouse No 17311. 0.18 M aqueous sol[illegible] [illegible]oxyethylene ether [illegible] 320 γ of DM[illegible] plastic IFE [illegible] stretched a[illegible] stickiness [illegible] [illegible] Note [illegible] though the dose of the compound is 1/100th of that in Fig 10. Hair follicular cycle: anagen. × 7,800

DISCUSSION

Exposure of the mouse back to laboratory-synthesized glyceryl-1-stearate-2,3-*bis*-polyoxyethelene ether (III) and to glyceryl-1,2-distearate-3-polyoxyethylene ether (IV), as well as, to some extent, to glyceryl-1,2-distearate (II) caused the interfollicular epidermis to develop the typical "Tween 60 effect" previously known from experiments with technical and laboratory-synthesized Tween 60 (polyoxyethylene sorbitan monostearate) Apart from the development of a regularly-built epidermal hyperplasia, this effect is characterized by maintenance of the ultrastructural patterns of the nucleated cells, including those of the mitochondria (3)

The biologic efficacy of these glyceryl stearate-type tumor enhancers depends on the agents themselves The following considerations support this view The agents employed were synthesized under the strictest control Addition of ethylene oxide chains to glyceryl-1-stearate (I) and glyceryl-1,2-distearate (II) increased both their tumor-enhancing property (1) and their ability to cause epidermal hyperplasia However, this mere addition process *did not* result *in* such untoward products as could be held responsible for these activities glyceryl-1,2-distearate (II), which contains no ethylene oxide, possessed both these biologic properties The electron-microscopic pattern essentially differed from that brought about by administration of carcinogen (4) Thus there seem to be no real grounds for regarding the "Tween 60 effect" as a results of exposure of the skin to hypothetical so-called borderline carcinogens or to other irrelevant by-products

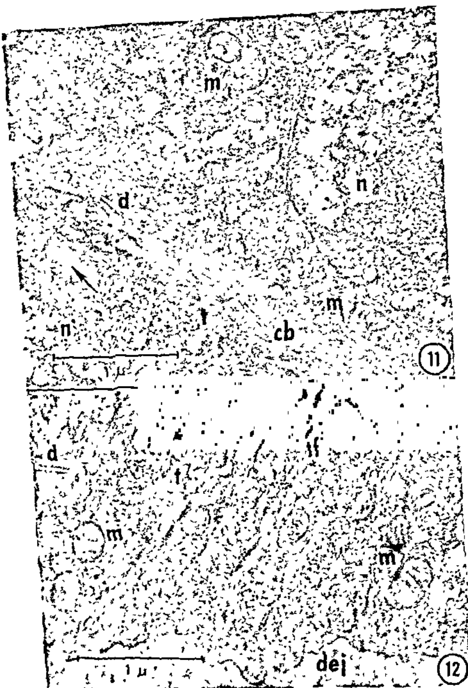
Accordingly, the group of agents known as tumor enhancers includes pure, synthesized substances whose chemical structure is fully known The search for new, chemically well-defined tumor enhancers for mouse skin with the frequent-application technique introduced in this laboratory has revealed the existence of a whole spectrum of them (*cf* discussion in (2, 9)) It has been predicted that such agents are

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Figs 11 12

Fig 11 Mouse No 17326 0 18 *M* aqueous solution of glyceryl 1 2 distearate 3 polyoxyethylene ether (IV) beginning 30 days after a single initial local application of 320 γ of DMBA 60th day an electron micrograph from the level of the upper pole of two adjacent basal cells of medium degree III hyperplasia The matrix of one of the mitochondria (*arrow*) appears electron dense Conspicuous is the good preservation of the general ultrastructural patterns of the cells in spite of the prolonged treatment Hair follicular cycle telogen × 39 000

Fig 12 Mouse No 17321 0 18 *M* aqueous solution of glyceryl 1 2 distearate 3 polyoxyethylene ether (IV) beginning 30 days after a single initial local application of 320 γ of DMBA 30th day an electron micrograph from the basalmost level of medium degree III hyperplasia *Note* again the rather good preservation of ultrastructural features Hair follicular cycle telogen × 39 000


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to be found particularly among the lipids, using this term in its widest sense (11, 12) So, for example, in frequent-application experiments after a single initial exposure to carcinogen, some fatty acids have proved to possess tumor-enhancing properties (13) as *Twort & Twort* demonstrated some 20 years ago (reviewed in (2)) Considering the large number of such substances, it is likely that new, well-defined tumor enhancers will be continuously discovered In addition, glyceryl-1,2 distearate, for instance, is a physiological substance

The difference between acetone and water solutions was interesting Just as in tumor-production experiments (1), in acetone nearly the same biologic response, *viz* hyperplasia of the interfollicular epidermis, was achieved with only about 1/90th to 1/100th of the dosage necessary in water This can be explained in terms of solvent function any disturbance of the lipid framework of the cells brought about by acetone either by precipitation or by dissolution of cholesterol from cellular interfaces breaks the barrier of skin absorption Further, it has been shown *in vitro* on various lipo'protein surface films (14) and with red blood cells (15) that aqueous solutions of non-ionic surface-active agents as such solubilize cholesterol from the phase boundaries When acetone is used as a solvent for glyceryl stearate-type agents, the solubilizing and/or precipitating effect increases On the other hand, when aqueous solutions of Tween 60-type agents are employed, these solutions have to act simultaneously both as solvents (mediators carriers) and as biologically active noxae Finally, considerable quantities of the non-volatile aqueous solutions are wasted through licking, rubbing and drying, which also helps to explain why such high doses are needed

The above results were obtained with the comparatively resistant mouse strain of this laboratory Entirely different responses are obtained with susceptible mouse strains (Swiss, CFW, CF # 1) that develop tumors even without preceding carcinogen initiation and that develop mitochondrial alterations in the IFE when the animals become old (to be published)

There is, then, a distinct difference between the behavior of the mitochondria of nucleated epidermal cells after exposure to dipole type tumor enhancers (*cf* also 3) and after exposure to carcinogens (4) Further, the keratin-stabilizing system of the epidermal cells after exposure to carcinogens appears inadequate, but quite normal after a similar exposure to Tween 60-type tumor enhancers (16) Finally it is known that the matrix of the normal mitochondria contains most of the enzymes of the citric acid cycle and that the enzymes of the respiratory cycle appear in the membraneous components Because of these reasons, there should be possibilities for study of the target(s) of the carcinogen effect within the cells of mouse epidermis with various biochemical techniques Such studies will be facilitated by the fact that during the normal differentiation process in kera-

tinizing tissue the structure including the ultrastructure (*i e* morphology) and the functions (*i e* physiology) seem largely to reflect on each other (*cf* conclusions in (2))

SUMMARY

The character of ultrastructural changes in mouse epidermis caused by laboratory synthesized glyceryl stearate type tumor enhancers in acetone and in water was studied The same material was examined histoquantitatively and by light microscopy

The general electron microscopic pattern agreed with the light microscopic appearance

The solvent acetone *vs* water was of importance In acetone related hyperplastic responses of the epidermis were achieved with only about 1 90th to 1/100th of the dosage necessary in water This may be understandable as the mammalian epidermis is not a water absorbing organ

Exposure of the skin to glyceryl 1 stearate 2 3 *bis* polyoxyethylene ether and glyceryl 1 2 distearate 3 polyoxyethylene ether and to some extent to glyceryl 1 2 distearate brought about ultrastructural patterns *in the epidermis* that were similar to those caused by technical and laboratory synthesized Tween 60 a high degree maintenance *of the* profiles of the nucleated cells including those of the mitochondria Exposure to these same compounds after initiation with 9 10 *dimethyl 1 2* benzanthracene caused changes that resembled those brought about with Tween 60 after similar carcinogen initiation

It was concluded that the biologic efficacy of these glyceryl stearate type tumor enhancers depend on the compounds *themselves* and not on irrelevant by products Thus the *group of* tumor enhancers for the mouse skin includes pure simple *synthesized substances* whose chemical structure is *fully known In addition* glyceryl 1 2 distearate is a physiological substance

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to be found particularly among the lipids, using this term *in its widest sense* (11, 12). So, for example, in frequent-application experiments after a single initial exposure to carcinogen, some fatty acids have proved to possess tumor-enhancing properties (13) as *Twort & Twort* demonstrated some 20 years ago (reviewed in (2)). Considering the large number of such substances, it is likely that new, well defined tumor enhancers will be continuously discovered. In addition, glyceryl-1,2-distearate, for instance, is a physiological substance.

The *difference between acetone and water solutions was interesting*. Just as in tumor-production experiments (1), in acetone nearly the same biologic response, *viz.* hyperplasia of the interfollicular epidermis, was achieved with only about 1/90th to 1/100th of the dosage necessary in water. This can be explained in terms of solvent function: any disturbance of the lipid framework of the cells brought about by acetone either by precipitation or by dissolution of cholesterol from cellular interfaces breaks the barrier of skin absorption. Further, it has *been shown in vitro on various lipoprotein surface films* (14) *and with* red blood cells (15) that aqueous solutions of non-ionic surface-active agents as such solubilize cholesterol from the phase boundaries. When acetone is used as a solvent for glyceryl stearate-type agents, the solubilizing and/or precipitating effect increases. On the other hand, when aqueous solutions of Tween 60 type agents are employed, these solutions have to act simultaneously both as solvents (mediators, carriers) and as biologically active noxae. Finally, considerable quan*tities of the non-volatile aqueous solutions are wasted through licking*, rubbing and drying, which also helps to explain why such high doses are needed.

The above results were obtained with the comparatively resistant mouse strain of this laboratory. Entirely different responses are obtained with susceptible mouse strains (Swiss, CFW, CF # 1) that develop tumors even without preceding carcinogen initiation and that develop mitochondrial alterations in the IFE when the animals become *old* (to be published).

There is, then, a distinct difference between the behavior of the mitochondria of nucleated epidermal cells after exposure to dipole-type tumor enhancers *(cf. also 3) and after exposure to carcinogens* (4). Further, the keratin-stabilizing system of the epidermal cells after exposure to carcinogens appears inadequate, but quite normal after a similar exposure to Tween 60 type tumor enhancers *(16)*. Finally it is known that the matrix of the normal mitochondria contains most of the enzymes of the citric acid cycle and that the enzymes of the respiratory cycle appear in the membraneous components. Because of these reasons, there should be possibilities for study of the *target(s) of the carcinogen* effect within the cells of mouse epidermis with various biochemical techniques. Such studies will be facilitated by the fact that during the normal differentiation process in kera

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STUDIES OF AN ABNORMAL γ GLOBULIN IN A CASE OF SUSPECTED MULTIPLE MYELOMA

By

S Eckerström S Glasse I A Hanson T Knutsen and I Brattsten

Received 5 ix 60

In a case of anemia in an old person the electrophoretic blood ana

tiple myeloma In our case the clinical symptoms did not allow an unambigous diagnosis of a disease of this kind although its presence in a latent form could not be excluded Analogous cases have been reported previously in which it could not be decided whether the paraproteinemias were multiple myelomas developing their clinical symptoms later or were related to other conditions (for example *Waldenstrom* 1952 *Kohn* 1960 *Heremans* 1960)

In cases of multiple myeloma the studies of the characteristic abnormal protein components which are frequently found in the blood have shown the general similarity of these proteins to the normal serum components of equal electric mobility For example in a series of investigations Kunkel and coworkers have been able to relate certain myeloma proteins to the typical γ globulins and others to the macroglobulin component which is a normal constituent of the β globulin group (*Kunkel et al* 1951 *Slater et al* 1955 *Muller Eberhard & Kunkel* 1956 1959) *Heremans* and coworkers (1959) have similarly shown the existence of myeloma proteins having the chemical physical and immunological characteristics of β x globulin which is also a normal serum component However the quantitative analyses carried out on myeloma proteins from various cases have revealed min ... ficant ...

pre

slig ... the composition—for example in the carbohydrate contents—may be responsible for the observed variations in the physical and immunological properties of these substances Physically the individual variation in the electric mobility is note

Financial support of the work from the Swedish Medical Research Council is gratefully acknowledged.

6 *Setala K Dammert K Setala H Merenmies I & Holsti P* Morphologische Hautveranderungen bei Mausen hervorgerufen durch nichtionisierbare *oberflachenaktive* tumorauslosende (tumour promoting) Substanzen Ztschr Krebsforsch *61* 548 568 1957

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15 interaction in monolayers II The agents on lipo protein mon layers

16 *Setala K Ayrapaa O Nisi men [illegible] nvall I & Nyholm M* Distribution of sulfhydryl and disulfide groups and birefringent material in mouse epidermis resulting from exposure to dipole tumor enhancers and carcinogens Naturwissenschaften 47 357 358 1960

There were no skeletal or joint pains nor did X ray examinations of the skeletal system show any signs of multiple myeloma There were no signs of cryoglobulinemia During the following six months the condition of the patient was essentially unchanged but after this time there was a slow remission and the anemia began to show improvement A new bone marrow biopsy still showed a slight increase of plasma cells but now also some polynuclear plasma cells thus providing a tentative myeloma finding

The condition of the patient slowly improved and she was discharged 14 months after admission in a rather good condition and with a Hb value of 12 7 mg%

CHEMICAL STUDIES

Preparations of the unknown component in the patient's serum were obtained by continuous zone electrophoresis (*Svensson & Brattsten* 1949 *Brattsten* 1955) The stabilizing medium consisted of cellulose powder When the protein concentration of the fractions had been recorded these were suitably combined the concentration of the resulting solutions adjusted to 1 per cent of protein by ultrafiltration, and the low molecular weight substances of the medium exchanged by dialysis Fig 1 shows the position of the abnormal protein component in the fractionation diagram The five fractions designated in the diagram were examined in the ultracentrifuge, analyzed chemically, and applied in the immunological investigation Similarly prepared fractions of normal serum were analyzed simultaneously for comparison Samples of the patient's serum were obtained for analysis in December 1958, February and April 1959 During this period no significant changes were observed

Physical characteristics of the unknown component The electric mobility of this component was in the range of $-2\,2 \cdot 10^{-5}$ cm² volt⁻¹ sec⁻¹ in a medium of veronal buffer of ionic strength 0 1 and pH 8 6 Electrophoresis at different pH reaction indicated that the mobility relative to the normal γ-globulin component remained constant in the range 4 5 to 9 5 Hence a differentiation of the abnormal component

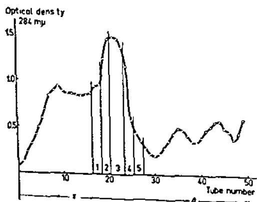


Fig 1

Representation of the analyzed fractions 1 5 They were obtained by electrophoresis of the patient's serum at pH 8 6 and ionic strength 0 027

worthy, as contrasted with the general constancy of the sedimentation constant The characteristic patterns obtained in immuno-analysis of serum *in cases of multiple myeloma has been studied in particular by* *Grabar* and coworkers (1956), *Korngold & Lipari* (1956), *Scheidegger & Buzzi* (1957) and *Heremans* (1960) These patterns demonstrate the similarities with the normal immune globulins and typical alterations induced by the pathological conditions as well

Present evidence thus indicates that the abnormal components appearing in the γ-, β_2-globulin fraction in serum in cases of multiple myeloma generally fall chemically into groups of known, normal serum globulins, yet exhibit considerable *individual variation from* case to case Under these conditions the chemical characterization of a substance suspected to be related to the disease will hardly provide information *of a direct, diagnostic value* Possibly a more conclusive import may be attributed to the immunological patterns inasmuch as the special features of these in reported cases of multiple myeloma appear to be rather uniquely associated with diseases of this type

REPORT OF CASE

An 82 year old woman was admitted to the Vasa Hospital of Goteborg for rehabilitation after a fracture of her right hip which had been operated upon 3 months before admission Physical examination on admission revealed a blood pressure of 210/125 The skin was pallid and there was generalized muscular weakness but she was able to stand and walk a little in a walkingchair No edema was present The [illegible] 10 5 mg% the total number of leucocytes was 3600/mm3 and the blood count [illegible] value was 26 per cent The F S R was [illegible] no albuminuria but a microscopic pyuria [illegible] the bacteriological examination showed the presence of *Proteus* bacteria *There were no signs of renal insufficiency*

As there was no response to iron therapy and the Hb was decreasing during the following month a bone-marrow biopsy was made showing a slight increase of the plasma cells but no atypical forms and no signs of a lack of an antipernicious factor In spite of only a slight increase of the F S R during the following month *(the maximum was 35 mm/1 h) a paper electrophoretic examination* of the serum was made The latter showed a pronounced increase of the γ globulins (table 1)

TABLE 1

Results of Paper Electrophoresis

Date	Tot *proteins* gm per cent	Albumin gm per cent	Globulins					
			α_1 gm p c	α_2 gm p c	β_1 gm p c	β_2 gm p c	γ^x gm p c	γ gm p c
1958								
23 XI	7 6	3 42	0 43	0 86	0 45	0 40	2 02	
29 XI	7 6	3 49	0 40	0 78	0 44	0 46	0 95	1 05
1959								
11 II	7 3	3 14	0 47	0 81	0 42	0 42	0 97	1 02
23 IV	8 0	3 83	0 37	0 74	0 61	0 37	1 01	1 05
13 VII	6 8	3 26	0 34	0 72	0 43	0 38	0 98	0 70
1960								
23 II	7 0	3 83	0 34	0 66	0 37	0 36	0 70	0 72

1948 *Svennerholm* 1956) using glucosamine as reference substance Sialic acid was estimated by the resorcinol hydrochloric acid reaction (*Svennerholm* 1957) The reference substance N acetylneuramic acid was a gift of Dr L Svennerholm Goteborg Phosphorus was determined according to *King* (1932) and nitrogen by *Markham's* method for micro Kjeldahl analysis (1942)

The phosphorus determinations gave values of 5 6 μg P/mg N in all the analyzed samples This is regarded as evidence that no protein containing phosphorus in significant amounts was present in the preparations

In the sugar analyses an attempt was made to exclude reducing material introduced in the course of the preparation by applying the procedure of *Goa* (1955) which employs precipitation of the protein by phosphotungstic acid prior to the hexose determination by the anthrone reagent Results comparable with those reported by him although generally lower were then obtained In particular it was found that the ratio of hexose to hexosamine in the fractions of the abnormal component 1 2 was the same as that obtained by him in a case of multiple myeloma (analysis of the γ globulin fraction) Since this procedure of hexose determination apparently gives too high values the analyses were repeated using direct reaction with the anthrone reagent and introducing a blank through all the steps in the preparation work The blank value was always less than 5 per cent of the sample value the contribution of material dissolved from the fractionation column was generally negligible The contents of hexose hexosamine and sialic acid in the preparations have been recorded in Table 2 Percentage concentrations of the three substances in the proteins have been computed from these data and included in the table The nitrogen contents in the proteins were assumed to be constant and equal to 15 6 per cent For the preparations of normal γ globulin the values obtained compare well with those recently reported by *Muller Eberhard & Kunkel* (1959) and *Heide* and coworkers (1959) In the case of the abnormal protein the carbohydrate concentrations are definitely higher than normal

IMMUNOLOGICAL STUDIES

Material

Antigens

1 The patient's serum
2 Samples of the abnormal component isolated by continuous electrophoresis (see above
3 Urine from the patient concentrated by pervaporation after dialysis
4 Normal human serum and preparation of normal human γ globulin isolated by the same procedure as the abnormal component
5 Samples of Bence Jones proteins belonging to groups I and II of *Burtin et al* 1956 kindly supplied by Dr Y Burtin Paris

Antisera

1 Rabbit immune sera were prepared against the isolated abnormal component by weekly injections of the preparation of this protein in a paraffin oil emulsion
2 Rabbit immune sera against normal human γ globulin (Behringswerke)
3 Rabbit horse and sheep immune sera against normal human plasma

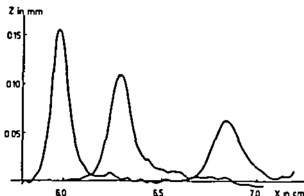


Fig. 2
Sedimentation analysis of fraction 3 from the patient's serum. The records were taken 20, 40 and 85 minutes after attainment of full speed.

from the normal γ-globulins was not obtained under the experimental conditions.

In the ultracentrifuge analyses the five fractions gave closely agreeing results, cf. Fig. 2.[1] In no instance could any trace of heavy material be detected ("18-S component", β_{2M}-globulin). An asymmetry in the boundary indicated some heterogeneity of the sedimenting material, in all the experiments approximately 85 per cent of the protein in the cell had a sedimentation constant of 6.5 S ($s_{20,w}$) under the prevailing conditions. These results were compared with sedimentation analyses of fractions of normal serum of corresponding mobility (*Brattsten* 1955). Except that a small portion of macroglobulin was observed in some of the latter experiments, the sedimentation characteristics of the unknown component were essentially similar to those of the normal material. The boundary generally exhibited the typical skewness and the sedimentation constant was the same, 6.5 S. This value was also obtained for the main portion of normal γ-globulin and coincides with that generally accepted. *Heremans* and coworkers (1959) report the same sedimentation constant for their preparation of β_{2A}-globulin. That protein gave a sedimentation pattern very similar to those obtained with our preparations (loc. cit., Fig. 3).

Chemical characteristics of the unknown component. In order to get some information of the chemical composition of the unknown component its contents of hexose, hexosamine, sialic acid, and phosphorus were determined with the experimentally found nitrogen value as the basis of the determinations. Corresponding fractions of normal serum were analyzed simultaneously.

Hexoses were determined by the anthrone method according to *Mokrasch* (1954) with an equimolecular mixture of galactose and mannose as reference, and hexosamine by means of Blix' modification of the method of Elson and Morgan (*Blix*

[1] Our thanks are due to Professor *Stig Claesson* and Miss *Anna Lisa Norling* of the Institute of Physical Chemistry, Uppsala, for facilities and aid in connection with the sedimentation analyses, which were carried out in the oil turbine ultracentrifuge of that Institute.

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IMMUNOLOGICAL STUDIES

Material

Antigens

1 The patient s serum
2 Samples of the abnormal component isolated by continuous electrophoresis (see above)
3 [illegible]
4 [illegible]
5 [illegible]

Antisera

1 Rabbit immune sera were prepared against the isolated abnormal component by weekly injections of the preparation of this protein in a paraffin oil emulsion
2 Rabbit immune sera against normal human γ globulin (Behringswerke)
3 Rabbit horse and sheep immune sera against normal human plasma

TABLF 2

Concentration of Hexose Hexosamine and Sialic Acid in Preparations of the Patient s Serum and Normal Serum

Fraction No	Preparations of the patient s serum							Preparations of normal serum						
	Hexose		Hexosaminé		Hexose/Hexosamine	Sialic acid		Hexose		Hexosamine		Hexose Hexosamine	Sialic acid	
	μg mg N	% in proteins	μg mg N	% in proteins		μg mg N	% in proteins	μg mg N	% in proteins	μg mg N	% in proteins		μg mg N	% in proteins
1	156	2 4	130	2 0	1 2$_{0}$	27	0 42	88	1 4	81	1 3	1 0$_{9}$	15	0 23
2	146	2 3	124	1 9	1 1$_{8}$	32	0 50	92	1 4	89	1 4	1 0$_{3}$	18	0 28
3	163	2 5	134	2 1	1 2$_{1}$	51	0 80	96	1 4	95	1 5	1 0$_{1}$	26	0 40
4	154	2 4	120	1 9	1 2$_{8}$	51	0 80	130	2 0	110	1 7	1 1$_{8}$	34	0 53
5	175	2 7	140	2 2	1 2$_{5}$	57	0 89	149	2 3	125	2 0	1 1$_{7}$	44	0 69

Methods

The immune electrophoresis of *Grabar & Williams* (1955) was used as modified by *Walsworth & Hanson* (1960) The identification experiments were performed by means of the comparative immune electrophoresis (*Walsworth & Hanson* 1960)

Results

Immune electrophoretic analyses of the patient's serum by means of the mentioned immune sera showed some irregularities of the γ globulin precipitate These were not very obvious, however, and were only revealed with some of the immune sera

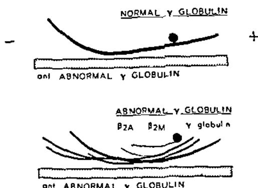


Fig 3

a) Immune electrophoretic analysis of normal γ globulin with the anti abnormal γ globulin serum showing a γ globulin precipitate of normal appearance
b) Immune electrophoretic analysis of the isolated abnormal γ globulin with the anti abnormal γ globulin serum The identified precipitates are designated

Similar analyses of the isolated abnormal component showed more apparent changes of the γ-globulin precipitate These were most evident when the analyses were performed by means of the immune sera against this isolated abnormal protein Five precipitation lines were then seen and three of them were located in the region of the abnormal fraction noted in paper electrophoresis (Fig 3 b)[1] One of them was crossed by the other two The latter two lines showed a partial crossing with each other The line nearest the immune serum basin showed two maxima one on each side of the partial crossing Furthermore, two faint precipitates were seen in the β_2-globulin region By means of the comparative immune electrophoresis the two latter lines could be identified as corresponding to the β_{2A} and β_{2M} globulins The other three lines all identified with the line formed by a preparation of normal γ globulin and anti-γ-globulin sera and with the γ-globulin precipitate in a human serum-anti serum spectrum All the antibodies in the im-

[1] A γ globulin precipitate of normal appearance was observed when normal γ globulin was analyzed with this immune serum (Fig 3 a)

mune sera against the abnormal protein could be completely absorbed with preparations of normal γ-globulins The urine sample contained proteins that identified with the serum γ-globulins of the patient One of the lines obtained with the abnormal protein identified with a Bence Jones protein of group I (*Burtin et al* 1956)

DISCUSSION

Since there is no indication that the normal proteins of electric mobility equal to that of the abnormal component are lacking or else fundamentally altered in the patient's serum, the preparations may tentatively be regarded as mixtures of the normally occurring globulins and the unknown component The analyses indicate a fairly uniform composition of the protein in the samples, which does not deviate appreciably from that of normal γ globulin The carbohydrate determinations show that the contents are higher than normal in the preparations of the patient's serum The concentration also increases slightly with (numerically) increasing mobility The observed elevation of the carbohydrate level may be correlated with the presence of β_{2A}-globulin which is richer in carbohydrate than the γ-globulins Both this component and β_{2M}-globulin have recently been reported to contain about 10 per cent of carbohydrate (*Muller-Eberhard & Kunkel* 1959, *Heremans et al* 1959) Hence, even a moderate proportion of either of these components in the examined preparations will influence the experimental values The sedimentation experiments give evidence that the macroglobulin component was not present in analytical amounts in the samples A differentiation between β_{2A}-globulin and normal γ-globulin is not possible by means of sedimentation experiments but the possibility that the former should be a major component in our preparations is ruled out by the chemical analyses, which indicate a lower carbohydrate content in the abnormal protein than would otherwise be expected These conclusions are in agreement with the immunological findings which show a fundamental relationship between the component under investigation and the normal γ-globulins Both β_{2M} and β_{2A}-globulin could be identified in the preparations by means of their specific precipitates

The immune electrophoretic pattern obtained in the analysis of the isolated abnormal γ-globulin fraction by its homologous immune serum indicated the presence of at least three proteins with different determinant groups, all reacting with anti-γ-globulin antibodies One of these proteins consisted obviously of two fractions with different electrophoretic mobilities, as suggested by the precipitate with two maxima The latter precipitation line showed a partial crossing with another line This gives evidence for the presence of a fourth determinant group Similar findings have been obtained with the abnormal serum proteins of patients with multiple myeloma in immune electrophoretic

analyses (*Grabar et al* 1956 *Scheidegger & Buzzi* 1957, and others) *Heremans* (1960) has observed such patterns also in denatured serum but in our case denaturation can probably be excluded as a cause of the results since the same pattern was obtained though less evident when fresh patient s serum was used as the antigen It may be noted that some of the used immune sera did not reveal any abnormalities of the patient serum or of the isolated fraction thereof The reason for this is presumably that these immune sera did not contain antibodies against all the determinant groups of the γ globulins Thus abnormalities of this type may well be overlooked if only such sera are available for the analyses Individual specificity has been reported for multiple myeloma proteins by *Slater et al* (1955) and by *Korngold & Lipari* (1956) and others In our case no such specificity could be demonstrated in the absorption experiments

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SUMMARY

In a case of anemia the results of paper electrophoretic investigations showed the presence of an abnormal serum globulin component in the patient s serum and bone marrow biopsies showed a picture that did not exclude the diagnosis of multiple myeloma A chemical and immunological study of the abnormal serum component was therefore undertaken

The chemical investigation showed that the unknown component resembles the normal γ globulins with regard to its carbohydrate contents and physico-chemical properties The immunological studies showed the presence of proteins with the same determinant groups as normal γ globulins The findings with regard to the carbohydrate contents and immuno precipitation patterns of the abnormal component were similar to those reported in cases of multiple myeloma

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SUMMARY

In a case of anemia the results of paper electrophoretic investigations showed the presence of an abnormal serum globulin component in the patient's serum and bone marrow biopsies showed a picture that did not exclude the diagnosis of multiple myeloma A chemical and immunological study of the abnormal serum component was therefore undertaken

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HISTOCHEMICAL INVESTIGATION OF SUCCINIC DEHYDROGENASE IN EXPERIMENTAL HYPOTHERMIA AND PARALLEL HISTO-PATHOLOGICAL OBSERVATIONS

By

L. LOFGREN

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When hypothermia was first taken into clinical use and its significance as a clinical adjuvant was fully realized, interest in the physiology of hypothermia increased greatly. The method proved to be risky, however, and for that reason studies of its pathologic anatomy came into the foreground.

Previous investigators (*Sano & Smith* 1940) were not able to establish any definite pathological changes except occasional focal pancreatic necrosis in patients with malignant tumours treated with hypothermia. Some other research workers (*Talbott, Consolazio & Pecora* 1941, *Delorme* 1952, *Bernhard, McMurrey & Curtis* 1955, *Bernhard, Cahill & Curtis* 1957) supported their statements, and up to the present time it has been considered that consistent or apparently significant pathological changes do not occur after experimental hypothermia in organs such as liver and heart (*Fischer, Fedor & Fischer* 1957). However, several authors (*Sarajas & Nilsson* 1954, *Knocker* 1955, *Sarajas* 1956, *Sarajas, Senning & Kaplan* 1956, *Heinrich, Holle, Schantz & Helpap* 1960, and others) have observed grave changes in the form of degeneration and focal necrobiosis.

Succinic dehydrogenase has been studied in liver and kidney of dog in connection with hypothermia tests (*Fischer, Fedor & Fischer* 1957). Decreased enzyme activity was noted in the liver after four hours' cooling, while in the kidney there was no change. As far as I am aware the heart has not been studied in this respect. — Reports in the literature at my disposal seem to be contradictory regarding the appearance of organic changes in connection with hypothermia tests. Considering their vital importance — especially in the heart — I considered it motivated to perform a histochemical study of the succinic dehydrogenase. This enzyme, so important in the biological oxidation process, seems

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to reflect damage of the organ of one kind or another before possible histological changes are established by means of routine methods and also to reach beyond the areas where histological changes arise (*Wachstein & Meisel* 1955, and others). Alongside with, and to complete the histochemical investigation, I made some histological examinations.

MATERIAL AND METHODS

The material comprises 12 adult male rabbits, unselected as to breed and age, apparently healthy, and weighing 2300 to 3100 g. The animals were given 30 mg/kg of Nembutal (pentobarbitone sodium) intraperitoneally. Fifteen to 30 minutes later the animals were immersed in an ice bath, the temperature varying between +2 and +8° C during the cooling period of the test. The body temperature generally fell from 28 to 25.1° C as recorded by a deep rectal thermocouple (Wallac-Thermex GS 2). After stopping the cooling process the animals' temperature decreased further by 0.3 to 1.3° C. Cooling to 18° C was done in one case. The animal was as a rule kept at a minimum temperature for at least one hour. Successive warming of the water bath to 40° C, followed by adding hot water, the animals were warmed to at least 38° C. Two of the animals were not re-warmed. Four animals were used for control; they were not cooled but given Nembutal. One of them died during the pre-test. Breathing was spontaneous during the hypothermia test.

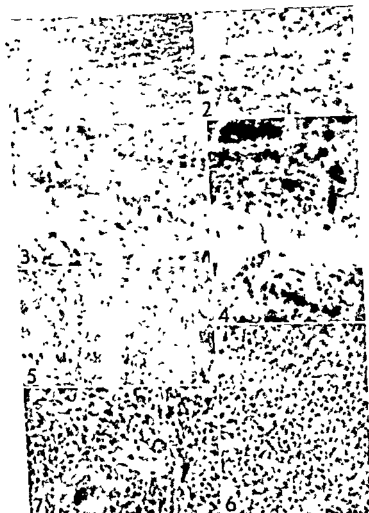
TABLE 1

Details of Hypothermia Tests

No.	Cooling temperature and cooling period			Temperature during at least one hour	Period of re-warming (to +38° C at least)		Duration of hypothermia	
3	28°.0	1 hour	20 min	27.2–27.6	1 hour	55 min	4 hours	20 min
6	27°.3	1	55	26.0–26.9	—		2	55
7	26°.1	1	30	25.8–26.8	2	35	5	5
8	18°.0	3	10	—	2	5	5	15 *
9	26°.8	2	15	25.9–26.7	2	45	6	5
10	25°.1	3	40	24.0–25.6	—		4	25
12	26°.3	2	10	25.3–27.2	3	5	5	15

* Given Nembutal 15 mg/kg after 2 hours and 15 min at a temp. 25.7.

All the rabbits were killed by a hit on the back of the head and immediately dissected. The heart was laid bare and removed. After gross study of the organ, pieces of the apical part of the left ventricle and often of the right one as well were cut out. Slices of the papillary muscles were also cut. *Seligman & Rutenburg's* (1951) histochemical method was followed for study of the succinic dehydrogenase. Neotetrazolium chloride (Nutritional Biochemicals Corporation) was employed as a staining indicator. Some of the preparations were immediately cut by means of a freezing microtome in serial sections of 15 µ. The sections were immersed in the incubation liquid and placed in a thermostat with a constant temperature of 38° C for two hours. After rinsing in aqua the sections were covered with glycerin and the cover glasses rimmed with paraffin. An adjoining part of the preparation was fixed in a 10 per cent formalin solution. Frozen sections of these were stained with Sudan IV for demonstration of lipids. Portions of each were further processed and embedded in paraffin in the usual manner. Sections of each specimen were stained with hemalum eosin, Weigert's hematoxylin van Gieson, Mallory's phosphotungstic acid hematoxylin, McManus' periodic acid Schiff method with and without prior digestion with malt diastase enzyme. All sections from each animal were treated and stained simultaneously.



Figs 1 7

Fig 1 Rabbit No 4 In the longitudinal slice of the muscle the formazan granules were arranged in regular longitudinal streaks 125 ×

Fig 2 Rabbit No 4 Enlargement of the same preparation 500 ×

Fig 3 Rabbit No 3 In the hypothermized animals, a striking abundance of conglo rations of the formazan granules occurred here and there 500 ×

Fig 4 Rabbit No 3 Conglomeration of formazan in the hypothermized animal 500 ×

Fig 5 Rabbit No 3 Formazan granules appearing here and there in sparce conglomerations Due to disorganization the longitudinal streaks have been effaced 500 ×

Fig 6 [illegible]

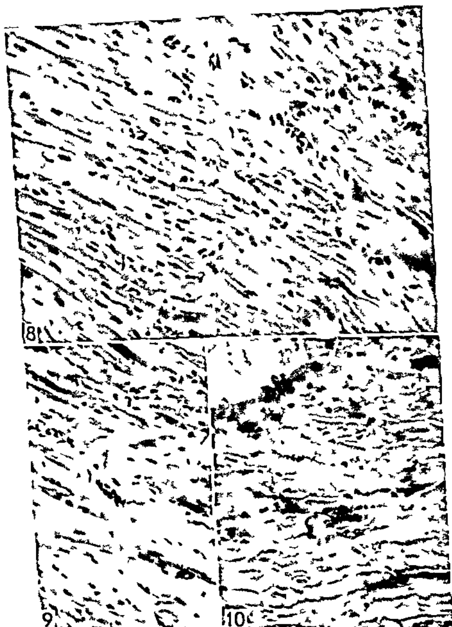
Fig 7 [illegible]

[illegible] granules 500 ×

RESULTS

Succinic dehydrogenase —The heart of the control rabbits revealed intense succinic dehydrogenase activity The reduced formazan granules in the longitudinal section of the muscle were arranged in streaks running lengthwise (Figs 1 and 2), and in the transverse sections, they were grouped like wreaths They were generally dark-blue-violet in colour, and almost black next to the endocardium and the epicardium, the membranes were free of enzyme, however, as were the vessels and connective tissue septa Small, solitary conglomerations of granules were seen here and there, but as a rule they were detached from one another In several of the hypothermized animals—but not in all – the formazan granules were more reddish and even pink in some parts, suggesting decreased activity (*Seligman & Rutenburg* 1951) by comparison with the controls The granules were further conglomerated in some parts (Figs 3 and 4) and much more so than in the controls Furthermore, it seemed as if in some parts, the formazan granules were farther apart (Fig 5) or closer together (Fig 6) than normal But above all, a disorganization of the regular longitudinal striations (Figs 3, 5, 6, 7) had taken place Especially in one of the animals treated with hypothermia (cooled to 18° C), there were, in addition to conglomeration and disorganization of formazan granules, clusters of rod shaped formazan granules surrounded by a reddish-violet area, resembling inclusion bodies (Fig 7) From what has been said one seems entitled to conclude that *in experimental hypothermia in rabbit, as performed in the present investigation, alterations are caused in the histochemically demonstrated succinic dehydrogenase* As these changes were evidenced also in the animals (Nos 6 and 10) that were not re-warmed, they should most closely be attributed to the hypothermia per se, and or hypoxemic hypoxia that arises during barbiturate anesthesia at spontaneous breathing, and not to the stress caused by heat stimulus during the re warming period This stress is not counteracted by barbiturate anesthesia (*Ronzoni* 1950) which is considered the case in connection with cold stress (*Sayers & Sayers* 1948, *Ronzoni* 1950, *Gellhorn & Redgate* 1951) Moreover, it should be mentioned that some authors propound the view that hypothermia as such is not a kind of stress affecting the corticosteroid activity (*Engdahl, Nelson & Hume* 1955, and others)

Histological findings – No signs denoting lesions appeared on gross inspection of the heart of the hypothermized animals The histological examinations performed alongside with the histochemical tests to establish if possible, pathological changes in the heart muscles arising in connection with the hypothermia tests yielded rather poor results With the knowledge of, for instance, *Sarajas* (1955) and *Heinrich Holle, Schantz & Helbig* (1960) having observed fairly regular pathological changes in experimental hypothermia tests on dog, it must be



F gs 8 11

F g 8 Rabb t No 7 Sub en locar i all there were small areas of ncreased eo ino phil a in patches and p s ibl ome p knotic nucle H + E 2 0 ×

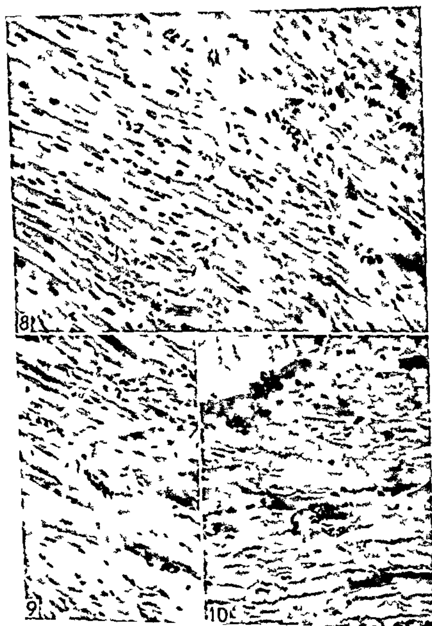
F g 9 Rabb t N 7 In the pa ts with incr ased e s n ph l a there was al o ncrea ed PAS c l rati n in patches PAS 2 0 ×

F g 10 Rabb t No 8 Area w th m re inten e PAS c l rati n PAS 250 ×

RESULTS

Succinic dehydrogenase.—The heart of the control rabbits revealed intense succinic dehydrogenase activity. The reduced formazan granules in the longitudinal section of the muscle were arranged in streaks running lengthwise (Figs. 1 and 2), and in the transverse sections, they were grouped like wreaths. They were generally dark-blue-violet in colour, and almost black next to the endocardium and the epicardium, the membranes were free of enzyme, however, as were the vessels and connective tissue septa. Small, solitary conglomerations of granules were seen here and there, but as a rule they were detached from one another. In several of the hypothermized animals—but not in all – the formazan granules were more reddish and even pink in some parts, suggesting decreased activity (*Seligman & Rutenburg* 1951) by comparison with the controls. The granules were further conglomerated in some parts (Figs. 3 and 4) and much more so than in the controls. Furthermore, it seemed as if in some parts, the formazan granules were farther apart (Fig. 5) or closer together (Fig. 6) than normal. But above all, a disorganization of the regular longitudinal striations (Figs. 3, 5, 6, 7) had taken place. Especially in one of the animals treated with hypothermia (cooled to 18° C), there were, in addition to conglomeration and disorganization of formazan granules, clusters of rod shaped formazan granules surrounded by a reddish-violet area, resembling inclusion bodies (Fig. 7). From what has been said one seems entitled to conclude that *in experimental hypothermia in rabbit, as performed in the present investigation, alterations are caused in the histochemically demonstrated succinic dehydrogenase*. As these changes were evidenced also in the animals (Nos. 6 and 10) that were not re-warmed they should most closely be attributed to the hypothermia per se, and or hypoxemic hypoxia that arises during barbiturate anesthesia at spontaneous breathing, and not to the stress caused by heat stimulus during the re-warming period. This stress is not counteracted by barbiturate anesthesia (*Ronzoni* 1950) which is considered the case in connection with cold stress (*Sayers & Sayers* 1948, *Ronzoni* 1950, *Gellhorn & Redgate* 1951). Moreover, it should be mentioned that some authors propound the view that hypothermia as such is not a kind of stress affecting the corticosteroid activity (*Engdahl, Nelson & Hume* 1955, and others).

Histological findings.—No signs denoting lesions appeared on gross inspection of the heart of the hypothermized animals. The histological examinations performed alongside with the histochemical tests to establish, if possible, pathological changes in the heart muscles arising in connection with the hypothermia tests yielded rather poor results. With the knowledge of, for instance, *Sarajas* (1955) and *Heinrich, Holle, Schantz & Helbig* (1960) having observed fairly regular pathological changes in experimental hypothermia tests on dog, it must be



F gs 8 11

F 8 h

F

F g 10 Rabb t No 8 Area w h more nten e PAS colo ation PAS 250 ×



Figs 11 12

Fig 11 Rabbit No 7 In one animal there were small sudanophilic drops in patches subendocardially within myocardial fibres Sudan IV 700 ×

Fig 12 Rabbit No 12 The cross striations were seen in areas with perivascular round cell infiltrations They were not visible where the PAS coloration was increased PAS 700 ×

said that on study of the present investigation, the very few and meagre findings made seem rather astounding It should be pointed out that the routine examination generally failed to reveal alterations Not until close study of the several slices had been made were some morphologic alterations observed, although not consistent ones, yet such that might be considered pathological In the preparations stained with hemalum eosin small patches with increased eosinophilia and possibly some pyknotic nuclei (Fig 8) were observed subendocardially These altered areas also presented increased PAS coloration in patches (Figs 9 and 10) to which the diastasis digestion did not contribute particularly Quite small perivascular round cell infiltrations (Figs 8, 9 12) were, however, seen mainly in the subendocardial muscles —It should be pointed out here that in one of the controls, I found a similar fairly large infiltration —The observation of such infiltrations appearing almost regularly has been specially mentioned by *Heinrich, Holle, Schantz & Helbig* (1960) in their studies of hypothermized dog They considered the originasation of these infiltrations due to disturbed permeability Sudan IV staining revealed the presence of fine sudanophilic droplets in patches within subendocardial fibers in one animal (Fig 11) No definite differences were observed in the cross striations of the altered areas as compared with the controls The cross striations appeared distinctly, for instance, in the areas with round cell infiltration (Fig 12) Van Gieson and Mallory s staining yielded no particular contribution

To sum up, it may be said that *in this study, the histological examination showed no consistent morphologic alterations that may be considered apparently significant for necrobiosis* The alterations appearing most often consisted of small perivascular round cell infiltrations *On the other hand, the histochemical examination revealed changes which*—as do previous investigations—*seem to precede those revealed by routine staining techniques* Viewed against this background, the fairly slight alterations in the histological picture noted in the present study, obtained a somewhat different significance The first signs of coagulative necrosis in experimental myocardial infarction are, for instance increased eosinophilia and increased PAS coloration, and pyknosis of nuclei (*Bing* 1956)

COMMENTS

The results show that experimental hypothermia in rabbit, such as used in this investigation, causes histochemically established alterations in succinic dehydrogenase To judge from the changes appearing (*Seligman & Rutenburg* 1951), the enzyme activity seems to decrease at times and in some parts of the heart muscle This phenomenon has been observed also in the liver of dog (*Fischer, Fedor & Fischer* 1957) Furthermore, during hypothermia, the formazan granules tend to con-

glomerate considerably more intensely than in the controls At the same time it seemed as if the granules appeared either further apart or more closely together than in the controls The most important fact was, however, that a kind of disorganization occurred, the regular longitudinal striations of granules appearing in the lengthwise section of the muscles were not visible As to the shift in the mutual position and organization of the granules, it may be said that the observations made agree well with what has previously been shown by several authors (*Zweifach, Black & Schorr* 1950, *Wachstein & Meisel* 1955, *Mustakallio, Saikkonen & Mustakallio* 1956, *Lofgren, Pettersson & Hjelmman* 1960) in heart and kidneys, and which has been attributed to hypoxia (*Lofgren, Pettersson & Hjelmman* 1960) In ischemic conditions there namely appears a tendency to conglomeration in the filamentous mitochondrias—the site of the enzyme—in the proximal convoluted tubules of the kidneys (*Emmel* 1940) "Many investigators have postulated that a reduced cardiac output and an increase of blood viscosity may cause capillary blood stagnation" in hypothermia, says *Gollan* (1959) The observation made by *Bigelow* and co-workers (*Bigelow, Lindsay & Greenwood* 1950) was, however, actually verified by *Gelin & Lofstrom* 1955, *i e*, that increased cooling brings about augmented conglomeration of erythrocytes in the peripheral blood flow, finally causing total stagnation of the capillary blood *Lynch & Adolph* (1957), also established that a capillary perfusion deficiency occurred due to total cessation of blood flow in the small vessels at low temperature It is known, moreover, that the blood pressure falls markedly during hypothermia (*Bigelow, Lindsay & Greenwood* 1950) This has been considered (*Sarajas, Senning & Kaplan* 1956) to lead to a fall below the "critical closing pressure" (*Burton* 1952) in some of the coronary branches It is further known that barbiturate anesthesia, as such, causes a reduction in tissue oxygen tension (*Larrabee, Garcia & Bulbring* 1952) If shivering sets in during the test—as was the case throughout in the present investigation the oxygen consumption increases to such a degree that the oxygen supply proves to be insufficient (*Gollan* 1959) Against this background, it by no means seems difficult to assume, like to *Sarajas* and co-workers (*Sarajas* 1956, *Sarajas, Senning & Kaplan* 1956) that relative hypoxia occurs in the cardiac muscles during hypothermia This occurs in spite of the oxygen supply during [illegible] (*Penrod* 1951, *Hegnauer* [illegible] [illegible]4) though it seems as if [illegible] [illegible]orm of muscular activity were lacking (*Edwards, Tulay, Reber, Siegel & Bing* 1954) This might be due to the hypoxia, however? The alterations observed in the succinic dehydrogenase supports the assumption in some degree As mentioned, changes of the same kind have been assumed to arise due to hypoxia

The hypothermia, as utilized in these studies, did not bring about

consistent morphologic alterations in the heart In some cases, there were subendocardial patches in the myocardium, with increased eosinophilia and possibly some pyknotic nuclei The same patches seemed to contain also an increased spot like PAS coloration One animal had sudanophilic droplets in some smaller areas in the muscle fibres The alteration occurring most often consisted in little perivascular round cell infiltrations—but it should be pointed out that a similar infiltration although larger, was seen also in one of the controls The difference in the appearance of apparently significant morphologic alterations in relation to those reported by other authors (*Knocker* 1955, *Sarajas* 1956 *Sarajas, Senning & Kaplan* 1956 *Heinrich, Holle, Schantz & Helbig* 1960) may probably be attributed to the different test animals and to the various test conditions In my investigation, the test animals were few in number, but hardly fewer than those cooled by *Heinrich, Holle, Schantz & Helbig* (1960) for 30 minutes only at a temperature below 30° C However, in these animals, they noted consistent pathological changes in the heart The procedure of taking only one or two sections of each heart for study may also have been a reason for the differences observed in this respect This technique seemed quite satisfactory for the histological examination made to complete the histochemical one The fairly slight morphologic changes appear, however, to obtain a wider scope when viewed against the background of the histochemical findings The last mentioned alterations are considered to precede the demonstrable morphologic alterations (*Wachstein & Meisel* 1955, *Ahlqvist & Telkka* 1957) The first signs of coagulative necrosis in experimental myocardial infarction are furthermore considered to be, for instance, increased eosinophilia increased PAS coloration and pyknosis of nuclei (*Bing* 1956) On the other hand, in this connection the almost completely negative results regarding morphologic alterations in the heart obtained in a large series of hypothermia tests (*Fischer Fedor & Fischer* 1957) should be borne in mind This divergence from the results of, for instance, *Sarajas* and co-workers (1956) was assumed to be due to the different technique of sampling used The morphologic alterations seen in the present study are fully motivated by what has been said in connection with succinic dehydrogenase Further studies are required, especially when some ECG examinations are taken into consideration *Sano & Smith* (1940), for instance observed no significant lesions in the heart of patients with electrocardiographic alterations during hypothermia *Santos & Kittle* (1958), also, established that the electrocardiographic changes in dog cooled to 26 27° C were chiefly of functional character and reversible But some other authors (*Heinrich, Holle, Schantz & Helbig* 1960) have observed in hypothermia tests, that the ECG changes often pers[illegible]

glomerate considerably more intensely than in the controls. At the same time it seemed as if the granules appeared either further apart or more closely together than in the controls. The most important fact was, however, that a kind of disorganization occurred, the regular longitudinal striations of granules appearing in the lengthwise section of the muscles were not visible. As to the shift in the mutual position and organization of the granules, it may be said that the observations made agree well with what has previously been shown by several authors (*Zweifach, Black & Schorr* 1950, *Wachstein & Meisel* 1955, *Mustakallio, Saikkonen & Mustakallio* 1956, *Lofgren, Pettersson & Hjelmman* 1960) in heart and kidneys, and which has been attributed to hypoxia (*Lofgren, Pettersson & Hjelmman* 1960). In ischemic conditions there namely appears a tendency to conglomeration in the filamentous mitochondrias—the site of the enzyme—in the proximal convoluted tubules of the kidneys (*Emmel* 1940). "Many investigators have postulated that a reduced cardiac output and an increase of blood viscosity may cause capillary blood stagnation" in hypothermia, says *Gollan* (1959). The observation made by *Bigelow* and co-workers (*Bigelow, Lindsay & Greenwood* 1950) was, however, actually verified by *Gelin & Lofstrom* 1955, i.e., that increased cooling brings about augmented conglomeration of erythrocytes in the peripheral blood flow, finally causing total stagnation of the capillary blood. *Lynch & Adolph* (1957), also established that a capillary perfusion deficiency occurred due to total cessation of blood flow in the small vessels at low temperature. It is known, moreover, that the blood pressure falls markedly during hypothermia (*Bigelow, Lindsay & Greenwood* 1950). This has been considered (*Sarajas, Senning & Kaplan* 1956) to lead to a fall below the "critical closing pressure" (*Burton* 1952) in some of the coronary branches. It is further known that barbiturate anesthesia, as such, causes a reduction in tissue oxygen tension (*Larrabee, Garcia & Bulbring* 1952). If shivering sets in during the test as was the case throughout in the present investigation the oxygen consumption increases to such a degree that the oxygen supply proves to be insufficient (*Gollan* 1959). Against this background, it by no means seems difficult to assume, like to *Sarajas* and co-workers (*Sarajas* 1956, *Sarajas, Senning & Kaplan* 1956) that relative hypoxia occurs in the cardiac muscles during hypothermia. This occurs in spite of the oxygen supply during

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SUMMARY

In this study of hypothermia tests on rabbits, alterations in the histochemically established succinic dehydrogenase were always seen The enzyme activity seemed to decrease in some parts of the heart Furthermore, the reduced formazan granules conglomerated in a higher degree than normally Disorganization occurred and the longitudinal striations visible in the lengthwise slices of the heart muscle disappeared

The morphologic alterations in the heart muscle examined by routine technique were scanty and not consistent In combination with the histochemically revealed changes, and against the background of these alterations, it seemed, however, as if myocardial injury had arisen although it could not be said that the changes were apparently significant The observations made support the earlier assumption, i e that the histochemically revealed alterations in the succinic dehydrogenase precede the morphologic changes revealed by routine methods The character and etiology of the alterations are discussed

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CHROMOSOME HETEROGENEITY OF CELL LINES IN VITRO

II Report of a Clonal Line of the Strain HeLa with Numerous Giant Cells[1]

By

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In a routine cultivated HeLa cell population, at a certain frequency cells occur which have a chromosome ploidy higher than the "normal" subtetraploid chromosome number of the stem-line forming cells (*Hsu* 1954). In the HeLa cell line grown in this laboratory, the peak frequency of these higher ploidies was found to be between 130 and 150, thus presenting about the double stemline chromosome number. It has been found that the frequency distribution of the chromosome numbers of HeLa cells changes after repeated feedings with 30 per cent active human serum in Hanks solution (*Saksela, Saxén & Penttinen* 1960 a and b). The most characteristic effect, and the one which appears first, is the almost complete disappearance of the cells with a higher chromosome ploidy. In studying the mechanism of this phenomenon, attempts were made to obtain clonal lines of these polyploid cells by means of the methods developed by Puck and his associates. As one of the established lines differed markedly from the others, and from the parental line, as regards its exceptionally wide variation of the chromosome number and its particularly low alkaline phosphatase activity, there seemed justification for presentation of the results.

MATERIALS AND METHODS

HeLa cells were used which had been cultivated by routine methods in 10 per cent heat-inactivated (30' 56° C) and filtrated human serum pool in Hanks solution. The cell inocula of about 100 cells/60 mm Petri dish were prepared according to *Puck et al.* The growth medium was the same as that employed in the routine cultivation and no agar was used in the Petri dishes. After about five hours of incubation in 5 per cent CO_2 atmosphere the cells had become attached to the glass surface, and the sites of cells which were certainly single were indicated at the bottom of the dish with special reference to those cells which appeared largest. The incubation was continued for about 10 days until distinct colonies had been formed. One colony from each dish was taken as follows. The dish was first rinsed thoroughly three

1 Aided by a grant from the Sigrid Juselius Foundation.
2 The technical assistance of Miss Dyweke Bremer is gratefully acknowledged.

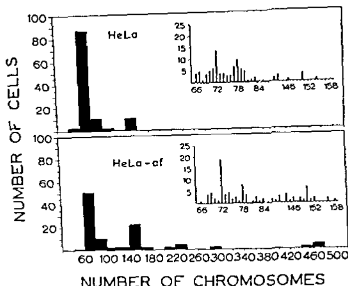


Fig 1
The frequency distribution of the chromosome numbers in the parental HeLa cell line and in the clonal HeLa cell line (HeLa af)

times with 5 ml of Hanks solution in order to eliminate those cells which might become detached from other colonies Under the microscope, the cells of the selected colony were detached with a sterile needle, dispersed carefully in 0 5 ml of Hanks solution and transferred with a pipette to a test tube Before a clonal line was established the cells [illegible]

The prepa[illegible] described by [illegible] reaction the [illegible] scopically w[illegible]

To measure the nuclear sizes, Feulgen stained preparations were projected on white paper the final magnification on paper being 1500 ×, and the images of the nuclei were drawn The areas of the nuclei were measured with 'Amsler Kompensations Planimeter' type 612

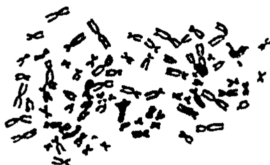
The reaction for alkaline phosphatase was carried out according to *Pearse* (1960)

RESULTS

The frequency distributions of the chromosome numbers of the parental HeLa cell line and the clonal line are presented in Fig 1 As can be seen, the frequency of the double stem-line chromosome numbers was greatly increased in the clonal line, and there occurred cells with a chromosome ploidy which was higher than in any of the cells of the parental line The difference in the chromosome pattern of the cell lines was found to be stable in subsequent counts during a period exceeding 3 months Fig 2 shows some typical sets of chromosomes of the clonal HeLa line

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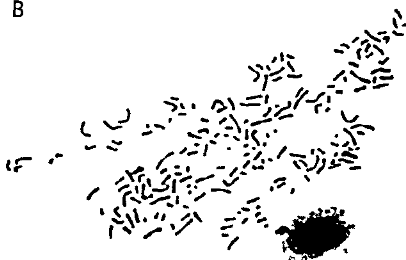




Fig 2

Fig 2

A set of 72 chro ... chr mos mes
C

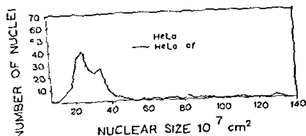


Fig 3
The d stribution of the nuclear sizes in the parental HeLa cell line and in the clonal HeLa cell l ne (HeLa af)

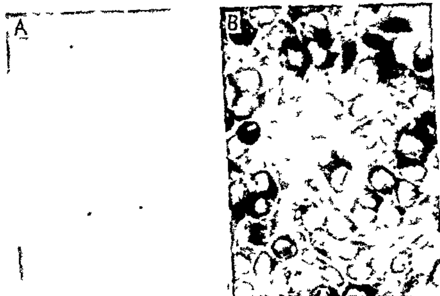


Fig 4
In A the alkal ne phosphatase activity in the clonal HeLa cell l ne is shown and B lemonstrates the same in the parental HeLa cell l ne (× 480)

tion of the nuclear sizes between the two cell lines are illustrated in Fig 3 A characteristic feature of the clonal line was also the appear ance of numerous multinucleated cells of which many showed great variation in nuclear size and form within one cell In some cells budd ing of small micronuclei from the giant nuclei was apparent Anomal

ous anaphase figures in the form of multipolar spindles and bridge formation were found, especially among cells of greater nuclear size

The alkaline phosphatase activity was found to be extremely low in all cells of the clonal HeLa cell line (Fig 4 a) In the parental HeLa, the activity varied from cell to cell, almost all cells with large nuclei showing particularly low alkaline phosphatase activity (Fig 4 b) This difference is discussed in more detail elsewhere (*Fortelius et al* 1960)

DISCUSSION AND SUMMARY

It might be supposed that the cell from which the clonal line started had been one of the double stem-line stock of the parental HeLa cell line This view is supported by the lack of the alkaline phosphatase activity from all cells of the clonal line, and its almost exclusive appearance only in the large cells of the parental line The wide heteroploid transformation which had occurred could be attributed to the various and rather common mitotic anomalies which have been described on HeLa cells (*Moorhead & Hsu* 1956, *Hsu & Moorhead* 1957), and of which some were also detected on the fixed preparations in this work Studies for further characterization of the clonal HeLa cell line are in progress For instance there seems to be some differences in the sensitivity to poliovirus type II between the two cell lines

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CELL RENEWAL IN THE WHITE AND BROWN FAT TISSUE OF THE RAT

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Received 25 xi 60

Little is known about the post natal renewal of fat cells. It has even been denied that any cell division at all occurs in the brown fatty tissue in the adult rat, in spite of apparent seasonal variations in its quantity (*v. Hansemann* 1902, *Coninx Girardet* 1927). After the administration of ^{14}C adenine and ^{14}C thymidine to adult mice, *Walker & Leblond* (1958) found autoradiographic evidence of an active cell renewal in different types of connective tissue, including the more specialized types such as fat cells. *Walker & Leblond* (1958) did not state however whether white or brown fatty tissue was studied or from what part of the body the fat was taken.

Our deficient knowledge of the growth and regeneration of the fatty tissue may be ascribed to the practical difficulties in identifying visually mitoses in cells with small and intensely stained nuclei (cf. *Leblond et al.* 1959). It may be expected therefore, that autoradiographic studies of the uptake of the DNA precursor, thymidine, should considerably add to our knowledge about cell division in fatty tissue, particularly as thymidine is now also available labelled with either tritium or radioactive carbon. The aim of the present investigation was to carry out, with the aid of tritium labelled thymidine, comparative studies of the cell proliferation in brown and white fatty tissue. White fat was taken from the subcutaneous gluteal deposits, while the brown fatty tissue was taken from the interscapular region.

MATERIAL AND METHODS

Rats of the Wistar strain wer...

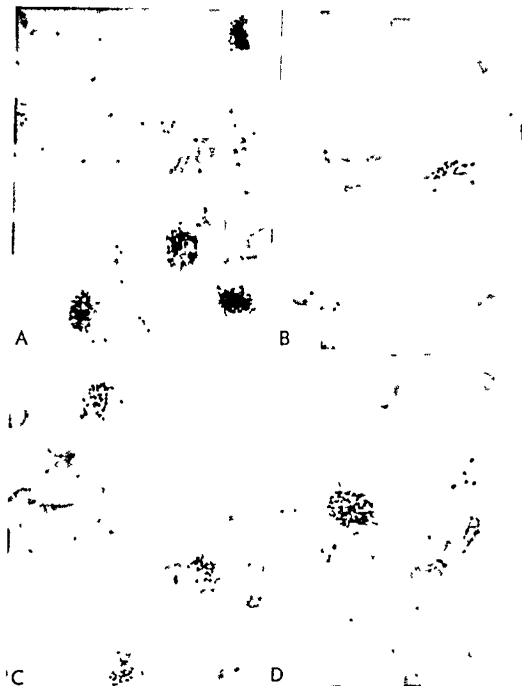


Fig. 1

A Subcutaneous fat from a 4 days old rat. Many strongly labelled cells were found. In a photographic reproduction the grain resolution is considerably reduced; even with direct microscopy, however, the number of grains over the most strongly labelled nuclei can only be estimated approximately. × 1400

B Subcutaneous fat from a 154 days old rat. The percentage number of labelled nuclei is low, and the average degree of blackening is lower than in the younger animals. A moderately intensely labelled nucleus may be seen in the central part of the picture. × 1400

C Brown fat from a 4 days old rat. As in the subcutaneous fat, many labelled cells were found. × 1400

TABLE 1

The Percentage Number of Labelled Cells in Brown and White Fat in Rats of Different Ages which were Injected with ^{3}H thymidine The Results have been given as Duplicate Determinations and their Means See Text for further Details

Animal no	Age in days	Brown fat			Subcutaneous fat		
		1	2	M	1	2	M
8000	4	35.7	36.6	36.2	51.3	54.0	52.7
8001	4	34.8	35.2	35.0	69.0	68.4	68.7
8002	11	53.0	50.4	51.8	68.9	69.3	69.1
8003	11	32.6	31.5	32.1	58.6	65.9	62.3
8004	22	32.0	31.2	31.6	42.3	38.8	40.6
8005	22	22.2	22.6	22.4	33.7	33.7	33.7
8006	32	22.6	24.7	23.7	15.7	20.0	17.9
8007	32	22.0	29.8	22.4	23.3	17.7	20.5
8008	42	20.8	21.7	21.3	18.2	20.0	19.1
8009	42	22.0	22.5	22.3	19.2	19.8	19.5
8010	53	18.0	17.3	17.7	19.7	21.9	20.8
8011	53	23.6	20.0	21.8	21.2	19.4	20.3
8012	63	17.6	21.1	19.9	21.0	21.1	21.1
8013	63	17.4	16.9	17.2	17.5	16.2	16.8
8014	73	–	–	–	13.3	14.2	13.8
8015	73	15.0	21.3	18.3	18.6	19.0	18.8
8016	95	15.3	17.7	16.5	9.0	10.5	9.8
8017	95	17.7	17.4	17.6	9.8	9.9	9.9
8018	154	15.0	12.9	14.0	8.8	9.8	9.3
8019	154		–		5.7	8.7	7.2
8020	154	12.6	13.7	13.2	–	–	–

8016) animal a classification of the degree of blackening was made by plotting the frequency distribution of the nuclei as a function of their grain content

RESULTS

Cells with distinctly blackened nuclei were found in all ages, both in the subcutaneous and brown fat (see Fig 1) The percentage number of labelled cells at different times after the administration of ^{3}H thymidine is given in Table 1 and Fig 2 For both types of fatty tissue the values tended to rise at first, and subsequently to decrease progressively during the whole observation period This decrease was greatest in the white fatty tissue While the frequency of labelled nuclei in this

Fig 1 (cont)

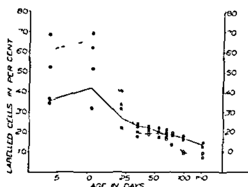


Fig 2

The percentage number of labelled cells at different ages in white (–O O–) and brown fat (■ ■–) The age of the rats in days is on a logarithmic scale The curve for the white fatty tissue is initially higher than the corresponding curve for brown fat, and subsequently slopes more steeply to fall below it

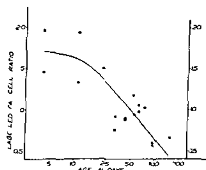


Fig 3

The ratio between the percentage numbers of labelled cells in white and brown fatty tissue in rats of different ages It may be seen that the ratio decreases with increasing age

type of fat corresponded initially to about 60 per cent, it was less than 10 per cent for the five month old rats The corresponding figures for the brown fat were, less than 40 per cent, and 10–15 per cent, respectively As a result of their different forms, the curves intersect at about the age of one month (see Fig 2) Fig 3 shows the characteristic decrease with increase in age of the ratio between the percentage numbers of labelled nuclei in the white and brown fatty tissue While the ratio thus at first exceeds 1 5, after about the age of two weeks it falls almost linearly down to about 0 5 in the last animals killed

The degree of blackening showed a progressive decrease during the period of observation The average number of grains was initially considerably higher in the white subcutaneous fatty tissue Figs 4 and 5, where for a young and an adult rat the frequency of cells is plotted in relation to the number of grains over the nuclei, illustrate both that the most intensely labelled cells are found mainly in the subcutaneous fat, and also that these cells are only found in the younger animal

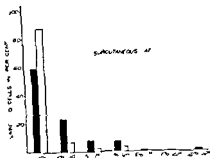


Fig 4

The frequency of labelled cells in white fatty tissue classified in relation to the number of grains over the nucleus The black bars refer to a young animal (4 days), and the white bars to an adult animal (154 days) The values given below the abscissa denote the number of grains for the class in question The percentage figures along the ordinate refer to the values in each class as a percentage of the total number of labelled cells

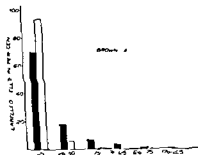


Fig 5

The frequency of labelled cells in brown fatty tissue plotted in relation to the number of grains over the nucleus The arrangement of the diagram is the same as in Fig 4

DISCUSSION

In interpreting the results, it must be recalled that DNA is stable, except in the pre-mitotic phase (*Brues, Tracy & Cohn* 1944, *Goldthwait & Bendich* 1952) The injected ^{3}H-thymidine therefore becomes incorporated only in cells which are about to divide Once a cell has been labelled it retains its radioactivity practically undiminished for a long period, provided that further division does not occur (*Leblond et al* 1959 *Diderholm & Hellman* 1960) It is furthermore of interest, that the period during which the injected thymidine is available for the DNA synthesis of the cells is relatively short *Hughes et al* (1958) found, for example, that after an intravenous injection of tritiated thymidine in mice, the thymidine had already left the circulation after a few minutes

There are thus good grounds for interpreting the high percentage

number of labelled cells in the animals killed first as an expression of the very active cell renewal at that time When a labelled cell divides mitotically, the radioactivity is distributed into two daughter nuclei, *i e.* the number of labelled cells increases With repeated divisions, the radioactivity gradually becomes so diluted that a number of the radio active nuclei are no longer recorded as labelled on the autoradiogram, *i e* the apparent frequency of the labelled nuclei is reduced The fact that after the 11th day of life there is a progressive decrease in the percentage of labelled nuclei which continues during the whole observation period, both in the subcutaneous and brown fatty tissue, thus shows, that neither of the two cell populations is stable, but are the seat of a continual cell renewal *The proliferation is probably greater* in the subcutaneous fat, since this curve falls more rapidly It should be emphasized in this respect, that this conclusion is based on the form *of the curve. The mere fact that the percentage number of labelled cells* was initially higher in the white fatty tissue, may not necessarily be a result of a greater cell renewal rate in the subcutaneous than in the *brown fat, but might be due also to the fact that the rate of synthesis* of DNA and the distribution of its precursor were different in the two types of fat

Since the values of each of the duplicate determinations were not very different, the random error in the determinations of the percentage frequency of labelled cells seems insignificant There were no cytological signs that the cells in the fatty tissue were damaged by the radiation The absence of very strongly radioactive cells in the oldest animals supports the interpretation that such cells in the young animals undergo normal mitotic division, with a subsequent dilution of the radioactivity

Until recently, the white fatty tissue was regarded as a rather passive storage organ for fatty acids synthesized in the liver Nowadays, however, it is becoming more evident that the fat cells take a very active part in the synthesis and break-down of fat By *in vitro* studies it has thus been shown that weight for weight the fatty tissue synthesizes at least 10 times as much fatty acid from glucose as the liver (*Hausberger* 1958) Our results indicate, that the fatty tissue should be conceived as a dynamic structure, not only as regards its metabolic activity, but also as concerns its rate of cell renewal In the post-natal growth of the subcutaneous fatty tissue, a direct renewal of fat cells, in addition to the fat storage in pre-formed cell elements is of considerable significance As regards the brown fatty tissue, there is a clear indication that, contrary to earlier contentions (*v Hansemann* 1902, *Gomnr-Girardet* 1927), cell division occurs there even in adults, although the rate of cell turnover is probably less than in the white subcutaneous fat

SUMMARY

At different times after the administration of tritiated thymidine the presence of radioactive nuclei was studied in brown and white fat with an autoradiographic technique In both types of fatty tissue the number of labelled cells tended at first to increase, but there was, subsequently a decrease which continued throughout the remainder of the observation period While the number of labelled cells in the white fatty tissue on the 4th day of life corresponded to about 60 per cent, this was less than 10 per cent in the 5 month old rats The corresponding values for the brown fat were about 35 per cent and 10–15 per cent respectively The results obtained show, that neither of the two fat cell populations is stable, but both are the seat of a continual cell renewal which seems to be more intensive in the subcutaneous fat

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A STUDY OF SOME PASTEURELLA STRAINS FROM THE HUMAN RESPIRATORY TRACT

By

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Received 25. xi. 60

Strains of the haemorrhagic septicaemia group of Pasteurella organisms are not common in the human respiratory tract, but a number of reports (2, 16, 18, 20, 24, 25, 27) show that P. multocida is occasionally isolated, both from cases of respiratory disease and from apparently healthy carriers (29, p. 1856). The considerable number of strains collected by some authors (20) might be thought to indicate that such organisms are less rare than commonly believed.

Previously one of us (S. D. H.) has had occasion to study three typical strains of P. multocida from the human respiratory tract, one (27) from an empyema of the pleura and two (unpublished) from the sputum of two patients, suffering from recurrent attacks of pneumonia. The latter cases may have been analogous to those described by *Olsen & Needham* (20). In the course of about two years 10 strains, apparently belonging to this genus, have been isolated from human nose or throat cultures. A preliminary report on three of these strains, believed to represent a new variety of Pasteurella haemolytica (var. ureae), has already been published (10). Some findings which were made in the course of a study of our strains, in particular the fact that they all gave a positive oxidase reaction, led us to extend the study to representative strains of several Pasteurella species.

MATERIAL

Strain NN was isolated from the nose of a man about whom we have no clinical information.

Strain 2175/58 was isolated from the nose of a 48 years old farmer who suffered from frequent colds. It was the only pathogenic species found in the cultures.

Strain 3520/59 was isolated from a 46 years old man living in a rural district who suffered from ozaena. Klebsiella ozaenae and atoxic Corynebacterium diphtheriae of the mitis type were also found in cultures from his nose.

Strain 218/60 was isolated from a nose culture of a 6 years old girl living in a rural district and suffering from acute rhinosinusitis. Large numbers of pneumococci and Haemophilus influenzae were also present.

Our thanks are due to Dr. *S. T. Cowan*, The National Collection of Type Cultures, London, and to Dr. *Ola Grini*, The Veterinary Institute, Oslo, for giving us strains of Pasteurella.

Strain 953/60 was isolated from the nose of a 16 years old boy It was present in large numbers together with some micrococci and diphtheroids

Strain 2885/60 was isolated from a nose culture of a 36 years old woman also living in the country and suffering from maxillary sinusitis The organism grew in large numbers in an apparently pure culture and might well have been the cause of the disease A second culture was unfortunately not obtainable

Strain S 2425/60 was isolated from a small boy with a retronasal fibroma, together with some ordinary commensals

Moraxella strain and the Pasteurella strain A few micrococci were also present A second nose culture taken about one week later yielded growth of the pasteurella

[illegible] colonies and some common throat organisms

The following representative Pasteurella strains were obtained for a comparative study

[illegible]

[illegible] From the Veter[illegible]

476/5[illegible]

culos

D

METHODS

Fermentation 1 per cent peptone water with 1 per cent sugar and Andrade's indicator Tests for gas production in glucose tubes covered with vaseline seals Some uncertain tests were repeated in the same medium after addition of 10 per cent ascites fluid and on ascites agar slants with sugar and indicator (phenol red)

Indol 1 per cent peptone (tryptic digest of casein) tested for indol by Ehrlich's reagent

Methyl red—Voges Proskauer reactions 4 days old cultures in 0 5 per cent peptone water with 0 5 per cent glucose Barritt's method for detection of acethylmethyl carbinol

Citrate utilization Koser's medium incubated for 4 days

Nitrate reduction 0 5 per cent peptone water with 0 ? per cent KNO_3 tested for nit[illegible]

and 3 days

for 10 days and checked

10 days

tr[illegible] days [illegible] tab with a loop and con

Oxidase reaction 3 different reagents were used

1 A c 0 5 per cent aqueous solution of tetramethyl p phenylenediamine hydrochloride prepared immediately before use

2 A 1 per cent aqueous solution of dimethyl p phenylenediamine hydrochloride, prepared in the same week

3 The test of Gaby and Hadly as used by *Ewing & Johnson* (7) Production of phenylpyruvic acid 2 drops of a dense suspension of organisms from agar slants in phosphate buffer of pH 6 8 were mixed with 2 drops of 0 1 per cent l phenyl alanine, incubated at 37° C for 2 h and tested for phenylpyruvic acid with 2 drops of half saturated $Fe(NH_4)(SO_4)_2$

Catalase test One drop of 10 per cent H_2O_2 was applied to agar slants

Many strains were unwilling to grow in some of the simpler fluid media Thus none of our strains nor the representative strains of P multocida P haemolytica or P pneumotropica would grow in the MR-VP medium, and the strains considered to belong to the two varieties of P haemolytica and to P pneumotropica failed to grow in some other media *e g* indol broth and nitrate broth In such cases the tests were repeated in media enriched with serum or ascites fluid

Antibiotic sensitivity tests The method of *Ericsson, Högman & Wickman* (6) Penicillin sensitivity was also tested in 10 per cent serum broth with the penicillin concentrations 1 – 0 5 – 0 25 – 0 1 – 0 05 – 0 025 units per ml The strains 3520/59 218/60, 3369/60 and 3974/60 and strain 12555 (P pneumotropica) grew too faintly for reliable readings

Pathogenicity 10 per cent serum broth cultures of our strains or heavy suspensions from blood agar cultures in the case of strains 3520/59, 218/60 3369 60 and 3974/60, were injected into guinea pigs in 0 5 ml volumes and intraperitoneally into mice in the doses 0 5 0 05 – 0 005 ml culture The strains 3520/59, 218/60 and 3369 60 were also injected into rabbits and mixtures of these organisms with mucin were injected intraperitoneally into mice

RESULTS

Morphology Only one gram-stained film of each strain, prepared from a 24 hour blood agar culture, was examined It appeared to be possible to refer the strains to a small number of reasonably distinct morphological groups The very striking appearance of group III may be emphasized This group comprises the 4 strains considered to be P haemolytica var ureae, as well as two reference strains of P haemolytica and 3 of P multocida Otherwise strains belonging to the various species are distributed on several morphological groups, with the exception of the strains of P pseudotuberculosis, which all were referred to group II, the one showing the "classical" morphology of Pasteurella (Table 1)

Colonies All our strains except 2 produced distinctly mucoid colonies, likewise 10 reference strains of P multocida and 3 of P haemolytica Typical S-colonies were produced by our strain S2425 60, by 4 strains of P multocida (948, 6535, 7707, 9659), 1 strain of P haemolytica (9380) and the strain of P pneumotropica The latter strain differed from the others in producing yellowish pigmented colonies Our strain 1225 60 produced colonies with a somewhat irregular, granular surface The organisms tended to agglutinate in saline, and in fluid media the strain grew with a fluffy deposit as long chains Therefore, the strain is considered to be in the R-form All 6 strains of P pseudotuberculosis grew as irregular, yellowish-grey colonies with a dull surface and of a very thin, watery consistency

Odor Cultures of our strains NN, 2175 58 952 60 2885 60, S2425 60 and 4225/60, all reference strains of P multocida and P pneumotropica produced the same very characteristic smell, which is very similar to

TABLE 1
Morphology of Pasteurella Strains Based on Examination of Gram Stained Film of 24 Hour Blood Agar Cultures

[illegible]	Description	Strains
I	Tiny coccoid cells apparently evenly stained but too small to discern structural details A few thin rods and short curved filaments	*AA 2175 58 2885/60 2425 60* P multocida *948 2417 3195 2026 55, 476/59 6 35 8747 11039 12945* P haemolytica 9712 P tularensis
II	Small plump oval coccobacillary cells of regular size many with distinct bipolar staining	*953 60* P multocida 1737 8565 P pseudotuberculosis 8315 8604 6904 6905 A 15 56 1933 56
III	Rather pleomorphic rods c 0 5 to 0 7 µ wide with rounded ends varying from short oval cells to short often curved filaments Many short rods show distinct bipolar staining Some long rods contain one or more unstained vacuoles	*3520/59 218 60 3369 60 3974 60* P *multocida 1876 7707 9659* P haemolytica 9380 T 20
IV	Short slender evenly stained uncharacteristic rods	*4295/60* P haemolytica T 18 P pneumotropica 12555

Strains isolated by us have been underlined

that produced by many strains of Haemophilus influenzae It has been stated in some papers that the odor of the cultures is not a reliable criterium of P multocida This is contrary to our experience with the strains available to us

All strains of P pseudotuberculosis produced a sharp somewhat disagreeable odor quite different from that of P multocida Our strains 3520 59 218 60 3369 60 and 3974 60 and the 4 strains of P haemolytica produced only a very faint fresh smell

Action on blood No genuine haemolysis was produced by our strains with the 4 exceptions noted below nor by the reference strains of P multocida P pseudotuberculosis or P pneumotropica A diffuse haemodigestion became apparent in cultures more than 24 hours old most strongly and most rapidly with cultures of P pseudotuberculosis

A group of strains comprising 3520 59 218 60 3369 60 3974 60 and the 4 reference strains of P haemolytica had a very characteristic appearance on blood agar with human or rabbit blood There was a very marked green discoloration usually accompanied by a slight partial haemolysis usually only a slight diffuse clearing of the medium but occasionally especially if the cultures were left on the bench for a few days is more distinct although very weak zones of haemolysis These changes most readily appeared when the cultures showed heavy, confluent growth whereas they might fail to appear when the strains grew as isolated colonies or appear late P haemolytica strain 9712 usually produced more marked haemolysis and less marked green color

than the others but even this strain produced only a very moderate partial haemolysis Although it is highly questionable whether it is correct to speak of these strains as haemolytic the changes produced are quite distinctive and probably of diagnostic value and the fact that 3 of 4 strains of P haemolytica showed exactly the same appearance is emphasized

Growth All strains of P pseudotuberculosis grew well on the usual media Strains NN 2175 58 953 60 2885 60 S2425 60 4225 60 and all strains of P multocida grew fairly well in most media but failed to grow on agar with a certain quality of peptone in the MR VP medium *and sometimes in nitrate broth*

Strains 3520 59 218 60 3369 60 and 3974 60 and the 4 strains of P haemolytica were more fastidious in particular our strains and grew scantily in fermentation tubes and not at all in the indol broth nitrate broth or the MR VP medium unless serum or ascites fluid was added

On MacConkey s agar all strains of P pseudotuberculosis grew well The 4 strains of P haemolytica grew very scantily one (T20) only in the second trial But all the remaining strains failed to grow

Blood agar cultures of most strains remained viable at room temperature for at least 10 days but strains 3520 59 218 60 3369 60 and 3974 60 often failed to grow in subculture from cultures more than 3 or 4 days old In some instances although growth failed when the subcultures were incubated in the incubator growth was obtained on parallel cultures incubated in a closed jar with a humid athmosphere Three of these strains were tested at various temperatures and grew at room temperature and at 37 C but not at 42° C

Biochemical reactions The results are shown in Table 2 Our strains NN 2175 58 and S2425 60 are quite typical strains of P multocida Strain 2885 60 differs from these only in fermenting lactose But the strains 953 60 and 4225 60 are typical in several respects They ferment maltose fail to ferment mannitol and sorbitol and split urea rapidly with a complete change of the indicator within 24 hours On the other hand many properties of these strains including morphology and most of the biochemical reactions are the same as those of P multocida and we therefore feel that these strains should be considered as strains of P multocida although they may represent a separate biotype or possibly a variety

The strains 3520 60 218 60 3369 60 and 3974 60 form a remarkably homogeneous group with quite uniform biochemical reactions as already reported (10) They are very like P haemolytica morphologically culturally and biochemically The only observed biochemical differences are the failure to ferment lactose and xylose and the very strong urease activity The decomposition of urea starts immediately after inoculation and a beginning change of the indicator color may be seen already after a few minutes

TABLE 2
Biochemical Reactions of Pasteurella Strains

Strains	Lactose	Mannitol	Maltose	Saccharose	Sorbitol	Dulcitol	Arabinose	Rhamnose	Xylose	Indol	M R	H_2S	Urease	Oxydase
NN		+		+	+	—	—	—	+	+	—	+	—	+
2175/58		+		+	+	—	—	—	+	+	—	+	—	+
2885/60	+	+		+	+		—	—	+	+	—	+	—	+
S 2425/60		+		+	+	—	—	—	+	+	—	+	—	+
953/60		-	+	+	-	—	—	—	+	+	—	+	+	+
4225/60	—	—	+	+	-		—	—	+	+	—	+	+	+
3520/59		+	+	+	+	—	—	—	—	—	—	—	++	+
218/60		+	+	+	+	—	—	—	—	—	—	—	++	+
3301/60	-	+	+	+	+		—	—	—	—	—	—	++	+
3974/60	-	+	+	+	+	—	—	—	—	—	—	—	++	+
P multocida 14 strains	—	+	—	+	+	3+	4+	—	8+	+	—	+	—	+
P haemolytica, 4 strains	2+	+	+	+	+	—	2+	—	+	—	—	2+	—	+
P pseudotubercul 6 strains	—	+	+	—	—	—	+	+	+	—	+	2+	+	—
P pneumotropica 1 strain	+	+	+	+	—	—	—	-	—	+	—	+	+	+

When reactions within a group of strains were variable, the number of strains giving positive reactions has been stated
These reactions were uniformly positive Fermentation of glucose and laevulose, reduction of nitrates, catalase These were negative
V P, citrate, liquefaction of gelatin or serum, production of phenylpyruvic acid

Of the 4 strains of P. haemolytica studied by us, two produced distinct, although weak acidity in lactose, and two (9380 and 9712) failed to ferment lactose, even on ascites agar slants. Three of 16 strains of P. haemolytica studied by *Newsom & Cross* (19) likewise failed to ferment lactose.

Strain 12555 of P. pneumotropica gave similar reactions, including a positive urease reaction, but it produced indol and did not give any haemolysis.

The oxidase reaction carried out with method 1 (tetramethyl-p-phenylenediamine) gave distinctly positive, although in some instances rather weak reactions with all strains isolated by us, all reference strains of P. multocida, P. haemolytica, P. pneumotropica and P. tularensis, but negative results with P. pseudotuberculosis. By method No. 2 (dimethyl-p-phenylenediamine) most strains of P. multocida showed a faint brownish color, which faded after a while, and which was considered as a negative reaction. The strain of P. tularensis gave a questionable reaction, and all other strains gave entirely negative reactions. By method no. 3 all strains considered to be P. multocida gave faint positive reactions in the form of light blue spots corresponding to the heaviest growth. These spots became apparent after 20–40 seconds and increased slightly within the observation period of 2 minutes, but remained weak and patchy. All the other strains were entirely negative. P. tularensis was not tested as it fails to grow on agar.

TABLE 3

Mouse Pathogenicity of 6 Strains of Pasteurella multocida by Intraperitoneal Injections of Serum Broth Cultures

Strain	Volume of cultures injected		
	0.5 ml	0.05 ml	0.005 ml
NN	2 d	2 d	2 d
2175/58	2 d	2 d	2 d
953/60	2 d	2 s	2 s
2885/60	2 d	1 d 1 s	2 s
S 2425/60	2 d	2 d	2 s
4225/60	2 d	2 s	2 s

Strains 3520/59, 218/60, 3369/60 and 3974/60 failed to kill mice except when mixed with mucin. d = died within 5 days. s = survived.

Pathogenicity. The results of pathogenicity tests on mice are shown in Table 3. Although the strains of P. multocida killed mice, only two showed more than a minimal virulence. The strains *3520/59, 218/60, 3369/60* and *3974/60* failed to kill mice, guinea pigs or rabbits (3974/60 was not tested on rabbits), even when large doses were given. The 3 first-mentioned strains were *also injected into mice with mucin* and then killed the mice, and the organisms were recovered from the heart blood.

TABLE 4

Antibiotic Sensitivity Tests on Pasteurella Strains by the Method of Ericsson et al (6) Grades of Sensitivity and Diameter (or Range of Diameters) of Inhibition Zones in mm

Strains	Sulphonamide	Penicillin	Streptomycin	Chloramphenicol	Oxytetracyclin	Erythromycin	Nitrofurantoin
NN	I (33)	I (31)	I (21)	I (27)	I (24)	II (21)	I (23)
2175 58	I (33)	I (33)	I (22)	I (32)	I (24)	I (25)	I (22)
2885 60	II (27)	I (31)	I (23)	I (33)	I (22)	II (23)	I (26)
S 2425 60	I (34)	I (30)	I (23)	I (38)	I (33)	I (26)	I (31)
953 60	I (45)	II (29)	I (29)	I (39)	I (27)	I (29)	I (35)
4225 60	I (32)	II (26)	II (17)	I (33)	I (26)	II (24)	I (30)
3520 59	I (37)	I (40)	I (27)	I (35)	I (25)	I (36)	I (30)
218 60	I (42)	I (33)	I (28)	I (40)	I (31)	I (32)	I (33)
3369 60	I (33)	II (25)	I (25)	I (32)	I (26)	I (26)	I (29)
3974 60	I (40)	I (31)	I (27)	I (38)	I (32)	I (28)	I (34)
P multocida 14 strains	I IV (0 38)	I II (28–35)	I–II (17–23)	I (24–41)	I (22 34)	I II (20–30)	I (20 44)
P haemolytica 4 strains	I (32 40)	II–III (20–28)	I II (17–20)	I (30 35)	I (27 30)	II (21–24)	I (28 33)
P pseudotuberculosis 6 strains	IV (0)	II III (16 29)	I (19 23)	I (23–30)	I (22 29)	III (12 15)	I (20 24)
P pneumotropica	I (41)	III (17)	I (27)	I (37)	I (29)	I (29)	I (33)
Lower limits of grades I II and III	28 22-16	30 21-11	19-15-11	21 15 10	20 16 8	25-19-11	15-

The grades used are I highly sensitive, II moderately sensitive III relatively resistant, IV resistant
The lower limits of these grades in mm diameter of inhibition zone are shown in the last line
The individual results obtained with our strains are shown but only summaries of the results obtained with the reference strains

In guinea pigs strain S2425/60 failed to produce any pathological change and 953/60 and 4225/60 produced only a transient local infiltration. 2175/58 and 2885/60 produced an extensive infiltration of the abdominal wall with prostration but the animals recovered. Strain XX likewise produced extensive infiltration of the abdominal wall and the animal succumbed on the 4th day. Thus the strains are not very virulent to guinea pigs.

Antibiotic sensitivity. The results of tests by the method of *Ericsson, Hogman & Wickman* (6) are shown in Table 4. It should be noted that many strains showed poor growth or failed to grow on peptone free horse blood agar and that therefore this medium was substituted by ordinary human blood agar with peptone. Due to this change some of the results obtained with sulphathiazole may be unreliable, particularly when the strains were found to give wide zones of partial growth inhibition. It should also be noted that a moderate variability of the results appeared when the tests were repeated. But these variations mostly were insignificant, although a few strains might be classified as highly sensitive to an antibiotic in one test and as moderately sensitive in another.

All strains were highly sensitive to chloramphenicol, oxytetracycline and nitrofurantoin and moderately or highly sensitive to streptomycin. The 6 strains of P. pseudotuberculosis were relatively resistant to erythromycin, whereas all other strains were highly or moderately sensitive. On the whole, sensitivity to penicillin was remarkably high for gramnegative rods, all strains except 4 being highly or moderately sensitive. The 4 exceptions were 2 strains of P. pseudotuberculosis (6904 and 6905), a strain of P. pneumotropica and one strain of P. haemolytica (T185). These strains were relatively resistant but still produced larger inhibition zones than the majority of other gramnegative rods.

The tests for penicillin sensitivity in serum broth gave the following results:

Of our 5 strains of P. multocida which were tested 4 were inhibited by 0.25 unit/ml and one (2885/60) by 0.1 unit/ml.

Of the reference strains of P. multocida 3 were inhibited by 0.25 unit/ml, 9 by 0.1 unit/ml and 2 by 0.05 unit/ml.

Of the reference strains of P. haemolytica 1 was inhibited by 0.1 unit/ml and 3 by 0.05 unit/ml.

Of 6 strains of P. pseudotuberculosis 1 was inhibited by 0.25 unit/ml and 5 by 1 unit/ml.

Strains 3520/59, 218/60, 3369/60 and 3974/60 did not grow well enough for reliable readings. This is also true of the strain of P. pneumotropica

DISCUSSION

The isolation of as many as 10 strains of Pasteurella from the human respiratory tract within a period of about two years shows that such organisms although rare in the human nose or throat occur frequently enough to be kept in mind It may perhaps be significant that no less than 5 of these strains were detected within a period of less than 3 months after we initiated this study As always special interest in a microorganism leads to more frequent isolation of it There is little doubt that many cultures which are discarded as without interest in the daily routine may contain such organisms which are mistaken for Haemophilus or Neisseria or some other common organism

The total number of nose and throat cultures examined within this two year period may perhaps be estimated to be somewhere between 3000 and 5000 and the frequency of isolated Pasteurella strains therefore is somewhere between 2 and 4 per thousand cultures

Of the strains 6 were classified as P multocida Three of these were typical in all respects and the fourth differed from the others only in fermenting lactose According to *Wilson & Miles* (29 p 880) strains isolated from buffaloes by *Tanaka* and from reindeer by *Magnusson* fermented lactose Two of 118 strains studied by *Rifkind & Pickett* (22) also fermented lactose

The two remaining strains were very like one another biochemically and they showed marked differences from the other strains in fermenting maltose failing to ferment mannitol and sorbitol and in hydrolysing urea *Meyer* (15) and *Wilson & Miles* (29 p 880) state that some strains of P multocida ferment maltose and *Meyer* states that strains isolated in some countries do not use mannitol

As for the urease reaction most strains appear to be negative as shown by *Morse & White* (17) and by our results But a strain isolated by *Lewis* (13) was urease positive and *Wilson & Miles* (l c p 881) state that P aviseptica may or may not decompose urea Therefore the differences between these two strains and the typical strains appear to be within the limits of variation of the species The fact that the reference strains of P multocida are so very homogeneous may be misleading since it is reasonable to suspect that these strains may have been selected just on account of their typical behaviour There may be reason to think that this collection of reference strains may be quite unsuitable to bring out the variability of the species

Therefore although we feel that the differences between our two strains and P multocida are insufficient to separate these strains in a taxon of their own it may still be pointed out that these strains represent another biotype than the reference strains and that future studies might show that they could be put in a separate variety of the species But for the time being they may better be considered as atypical strains of P multocida

Of these 6 strains, it is highly probable that one (2885/60) was the cause of the bilateral maxillary sinusitis. Two strains (2175/58 and 953/60) may have been the cause of similar infections, since they were the only pathogen found and were present in large numbers, but our information is insufficient for definite statements. One strain (S2425/60) was obviously not the cause of disease, as the patient did not suffer from an infection, and the role of the two remaining strains is unknown.

P. multocida has repeatedly been found as the cause of respiratory infection (2, 16, 18, 20, 24, 25, 27), but its isolation from healthy carriers apparently has been reported only by *Wilson & Miles* (29, p. 1856), unless some of the patients studied by *Olsen & Needham* (20), and similar cases reported by others (16) should be considered as healthy carriers.

The 4 remaining strains are believed to represent a new variety of P. haemolytica, *var. ureae*, as already reported. Although considerable numbers of colonies were found in each of these cases, the pathogenic role of these organisms is questionable and has not yet been demonstrated. Three strains were isolated from patients suffering from rhinosinusitis, but in two of these cases other pathogens, which are common causes of such infections, were also present. In the third case a mixture of the pasteurella and a strongly mucoid strain of Moraxella nonliquefaciens was found in the nose, but in pus from the maxillary sinus only the latter organism was found, microscopically and by culture. In this case a second nose culture, taken about one week later, yielded growth of the same pasteurella strain. In the fourth case, finally, the organism was found mixed with a typical ozaena flora.

These strains were exactly like one another in all characteristics that were studied, and they also were very like the strains of P. haemolytica. Morphologically all our strains were very characteristic and exactly like two of the strains of P. haemolytica. The morphology is so characteristic that the presence in the nose culture of the last strain to be isolated, was suspected after examination of a gram-stained film. But for this finding, the colonies of the strain would probably have been confused with the, likewise mucoid and more conspicuous, colonies of Moraxella.

Our strains differed from the reference strains in failing to ferment lactose and xylose, and to grow on MacConkey's medium, in being more difficult to cultivate in some fluid media and in the extremely rapid decomposition of urea. Of these differences, failure of lactose fermentation can hardly be considered as very important in view of the fact that two of the strains of P. haemolytica failed to ferment lactose. *Newsom & Cross* (19) also encountered such strains. Xylose fermentation may possibly be a more constant characteristic of P. haemolytica (3, 19).

Ability to grow on MacConkey's agar appears to be an important characteristic (22, 28). *Rifkind & Pickett* even consider this property

and lack of indol production as the two most important criteria in the differentiation between P haemolytica and P multocida

Therefore, the failure of our strains to grow on this medium seems to be an important difference It must be admitted, however, that none of the 4 reference strains of P haemolytica showed more than a scanty growth Indeed one of them failed to grow in the first attempt But even so there was a distinct difference from our strains since none of them produced a trace of growth

The most striking difference between our strains and the reference strains, however, is the strong urease activity of our strains The 4 reference strains did not split urea, in agreement with the experience of *Morse & White* (17) with 5 strains

As already stated (10) we feel that these differences may be insufficient to justify the creation of a new species, but that a new variety of the species should be created, *P haemolytica var ureae* The variety containing the type of the species then becomes *P haemolytica var haemolytica* As the type culture of the new variety we designate the first strain to be isolated, 3520 59 This strain has been deposited in the National Collection of Type Cultures, London, where it has been given the number 10219 (5)

The action of our strains on blood agar deserves comment When the strains grow with few, well separated colonies, there may at first be no apparent change, and only gradually diffuse greenish zones with little haemolysis appear around the colonies When growth is heavy and confluent on the other hand, the green color is very conspicuous even in cultures less than 24 hours old The haemolysis is slight, only a partial haemolysis with diffuse limits, of a similar appearance as that produced by some alpha streptococci It does not look like true haemolysis produced by a soluble haemolysin, but may be only a partial destruction of the erythrocytes caused by some other chemical activity of the organisms

Three of the four reference strains of P haemolytica produced similar changes on blood agar with human or rabbit blood The 4th strain (9712) produced more distinct zones of haemolysis and usually less green color

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th [illegible] media but variations have also been noted between different strains studied by the same investigators *Jones* (12) stated that deep colonies in 8 per cent horse blood agar were "surrounded by a clear zone of haemolysis" Nothing was said about haemolysis of surface colonies *Tweed & Edington* (28) found their strains to be haemolytic on blood agar, but no haemolysis was produced in blood broth *Newson & Cross* (19) also found their strains to be haemol[illegible] blood agar [illegible]

tion the green color which was so conspicuous in our cultures If this is a constant characteristic and can be confirmed in other laboratories and on media of different composition, it would be a just as useful characteristic *in recognising these organisms,* as haemolysis

We have not found any reference to isolation of P haemolytica from human sources in the literature Indeed, in the latest edition of Zinsser's Microbiology (26, p 468) it is stated· "There are no reports of human infections with this organism, but they may be expected to appear as clinical laboratories develop more interest in the Pasteurella group of organisms"

It may be expected that future studies by medical and veterinary bacteriologists will provide information on the habitat of this new variety and will show whether it causes infections in animals

The study of the biochemical reactions of the pasteurella strains gives some new information Thus it is shown that none of the strains decompose phenylalanine with production of phenylpyruvic acid More *interesting are the results of the oxidase tests Although* we have surveyed much of the recent literature on Pasteurella, including a number of textbooks and reference books (3, 4, 8, 15, 21, 26, 29), we have not been able to find anything about the oxidase reaction of Pasteurella species Possibly such tests have not yet been made According to our results distinct, although not always very strong, oxidase activity can be detected in all strains of P multocida, P haemolytica var haemolytica and var ureae, and in the single strains of P tularensis and P pneumotropica, by means of the most sensitive reagent, tetramethyl p phenylenediamine By the method of *Gaby & Hadley* only the strains *of P multocida showed a trace of oxidase activity, whereas the results* obtained with dimethyl-p-phenylenediamine solution were negative in all tests These results add several new species to the list of organisms producing oxidase In a previous paper one of us (9) made the erroneous statement that oxidase was not known to be regularly produced in any bacterial genus except Neisseria and Moraxella *Although it may* be true that the oxidase reaction is stronger, and therefore detectable by comparatively insensitive reagents, in these two genera than in most others, it has been shown as early as 1929 (14) that several other organisms produce oxidase several species of Pseudomonas, Brucella, Vibrio, Spirillum, Alcaligenes as well as Bacillus *Ewing & Johnson* (7) confirm that Vibrio, Pseudomonas and Alcaligenes species give the reaction, and add Aeromonas to *the list*

It is of special interest that there is agreement in this respect between several Pasteurella species, Brucella and Moraxella all considered to belong to the same family It would be of considerable interest to study this reaction in the groups of organisms belonging to this family, which have not yet been studied *sufficiently thoroughly It is also interesting that* P pseudotuberculosis differs from the other species, and it would be of considerable interest to check the behaviour of P pestis

The oxidase reaction is an interesting reaction which may not yet have been fully utilised in bacterial classification and it would be useful if this reaction could be used on organisms which have not yet been tested It seems that there is a very considerable difference between various oxidase tests and that tetramethyl p phenylenediamine is a more sensitive reagent than the others in current use

The results of the antibiotic sensitivity tests show that these Pasteurella strains as a group are more sensitive to penicillin than the majority of gram negative rods The strains of P pseudotuberculosis tended to be rather less sensitive than P multocida and the two varieties of P haemolytica and there is a similar difference with respect to sensitivity to erythromycin

SUMMARY

Ten strains of Pasteurella were isolated from the human respiratory tract 9 from nose cultures and 1 from a throat culture

Six strains were classified as P multocida Of these 3 were typical in all respects one fermented lactose and two gave several atypical reactions and may belong to a different biotype than the others

Four strains were assigned to a new variety of P haemolytica *var ureae* The type strain of this variety (3590 59) has been deposited in The National Collection of Type Cultures London where it has received the number 10219 The pathogenic role and habitat of this organism are yet unknown

All strains of P multocida P haemolytica var haemolytica and var ureae P tularensis and P pneumotropica gave a positive oxidase reaction with tetramethyl p phenylenediamine All strains of P pseudotuberculosis were negative None of the Pasteurella strains decompose phenylalanine to phenylpyruvic acid

All strains were comparatively sensitive to penicillin and erythromycin but the strains of P pseudotuberculosis tended to be slightly less sensitive in particular to erythromycin than the others

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TECHNIQUE OF METHYL RED TEST

By

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The importance of a uniform and correct technique for the IMViC test was pointed out in a previous paper (*Ljutov* 1959) which also contained a study of the indole test. The present paper will deal with the methyl red test (MR).

Clark & Lubs (1915) stated that the method proposed by them for the MR test was based on Smith's observation that bacteria of the coli-aerogenes group have the ability to form principally two gases, CO_2 and H_2. The phenomenon was later studied by *Rogers, Clark, Davis & Evans*, who found that coli-aerogenes bacteria may be divided into two groups on the basis of the ratio of CO_2 to H_2 which they produce. One group, the low ratio organisms, produces carbon dioxide and hydrogen in the constant ratio $CO_2/H_2 = 1.06$ and derives from faeces. The other, the high ratio group, which derives from grain, furnishes a ratio which varies between 1.90 and 3.00. In subsequent studies *Clark & Lubs* showed that the production of gas in different ratios is related to the pH value of the cultures, the low ratio organisms having lower and the high ratio organisms higher pH values. To avoid the necessity of determining the gas ratio and of electrometric determination of pH of the cultures, *Clark & Lubs* suggested the addition of methyl red solution as indicator. Lower pH values give a positive (red) and higher a negative (yellow) reaction.

MATERIAL AND METHODS

The material consisted of 613 coliform strains, 483 from 59 well waters and 130 from faeces. The strains were examined by the IMViC test for their ability to produce gas and acid from lactose, lactose peptone broth tubes being incubated at eleven temperatures between 22° and 45° C (see *Ljutov* 1959). The material is listed in detail in Tables 1 and 2.

The dextrose phosphate medium contained 0.5 per cent dextrose, 0.5 [illegible]
peptone 0.5 per [illegible]
After cultivation [illegible]
carboxylic acid) [illegible]
produced a rathe[illegible]
methyl red in 9[illegible] & Lubs solution (1915) and (3) 0.04
per cent sodium methyl red in 95 per cent alcohol.

TABLE 1

Typing of 483 Coliform Strains from 59 Well Waters

Type	IMViC	Number of strains able to produce gas up to											Total	Grand total	%
		22°	27°	30°	33°	35°	37°	39°	41°	43°	45°	46°			
E. coli	+ + − −			1		2	−	2	1	2	74	14	96	120	24.9
	+ −		−	−	−		9	2	3	4	3	3	24		
Interm	+ + − +					−	4	4	4	1		1	14	132	27.3
	− + − +		−	1	1	11	21	25	14	21	3	−	97		
	+ − + −		−	10			1			−	−	−	11		
	− − + −			2	2	−	4	1	1	−	−	−	10		
B. aerog	+ − + +	1	2	−	2	4	6	11	10	8	2	−	46	199	41.2
	− − + +		1	1	1	18	65	30	6	19	12		153		
Irreg	− + + +				1	3	−	4	2	−	−	−	10	32	6.6
	+ + +			1	1	3	7	2		5	−	−	19		
	+ + + −				1	−	−	−	−		−	−	1		
	− + + −	−		−		1			−	−		−	1		
	+ − − +				−	1	−	−		−	−	−	1		
Total		1	3	16	9	43	117	81	41	60	94	18		483	100

TABLE 2

Typing of 130 Coliform Strains from Faeces

Type	IMViC	Number of strains able to produce gas up to				Total	Grand total	%
		41°	43°	45°	46°			
E. coli	+ + − −		3	58	44	105	113	87.0
	− + − −	−	−	4	4	8		
Interm	+ + − +	1	1	−	−	2	10	7.7
	− + − +		3	1	−	4		
	+ − + −	1	3	−	−	4		
B. aerog	+ − + +	2	3	−	−	5	5	3.8
Irreg	+ + + −		−	1	−	1	2	1.5
	− + + +	1	−	−	−	1		
Total		5	13	64	48		130	100.0

EXPERIMENTAL

Incubation temperature To determine the optimal incubation temperature for cultivation of coliform bacteria for testing for MR reaction material was collected from agar slants of 613 coliform strains. The material was inoculated into dextrose phosphate tubes which were incubated at 22°, 27°, 30°, 33°, 35°, 37° and 39° C for three days.

It was found that, at 39° C, 101 strains did not grow and 25 strains, owing to very weak growth, yielded a negative MR reaction. Incubation at 39° C was therefore excluded from further study. Nineteen strains showed no visible growth at 37° C, but at 35° C and below all 613 strains grew well.

Examination of 613 cultures at 22°–37° C showed that 104 strains had a negative and 383 strains a positive MR reaction at all incubation temperatures from 22° to 37° C. Let us call these "stable" strains. Varying reactions were afforded by 126 strains, 125 of which from well water (25.9 per cent of 483 strains investigated), and one from faeces (0.8 per cent of 130 strains), the reactions being negative at low but positive at high incubation temperatures (see chart 1). Let us call these "unstable" strains. At the first examination for MR reaction after isolation 126 strains showed the following numbers of negative reactions: 126 strains at 22°, 125 at 27°, 106 at 30°, 59 at 33°, 20 at 35° and no strain at 37° C.

The threshold temperature between negative and positive reactions varied, however, in individual "unstable" strains on different occasions. At a second investigation the same 126 "unstable" strains showed the following numbers of negative reactions: 110 strains at 22°, 105 at 27°, 94 at 30°, 65 at 33°, 32 at 35° and 13 at 37°, while 16 strains

TABLE 1

Typing of 483 Coliform Strains from 59 Well Waters

Type	IMVC	Number of strains able to produce gas up to 22°	27°	30°	33°	35°	37°	39°	41°	43°	45°	46°	Total	Grand total	%
E. coli	+ + − −			1	−	2	−	2	1	2	74	14	96	120	24 9
	− + −	−			−	−	9	2	3	4	3	3	24		
Interm	+ + − +		−	−			4	4	4	1	−	1	14	132	27 3
	− + − +			1	1	11	21	25	14	21	3	−	97		
	+ − + −		−	10		−	1		−	−	−	−	11		
	− − + −		−	2	2		4	1	1	−	−	−	10		
B. aerog	+ − + +	1	2		2	4	6	11	10	8	2	−	46	199	41 2
	− − + +	−	1	1	1	18	65	30	6	19	12	−	153		
Irreg	+ + + +			−	1	3	−	4	2		−	−	10	32	6 6
	− + + +		−	1	1	3	7	2	−	5	−	−	19		
	+ + + −			−	1	−	−	−	−	−	−	−	1		
	− + + −	−	−		−	1		−		−	−	−	1		
	+ − − +		−	−		1	−		−	−	−	−	1		
Total		1	3	16	9	43	117	81	41	60	94	18		483	100

yielded positive reactions between 22° and 37° C The stable strains, on the contrary, yielded uniform reactions on all occasions

As seen from Table 3, 84 1 per cent of unstable strains were B aerogenes and 15 9 per cent intermediaries Among 224 strains capable of producing gas up to 45° C, only one was unstable (0 4 per cent) Of 389 strains which produced gas only at temperatures below 45° C, 125 were unstable (32 per cent)

Incubation period For determination of the most suitable incubation period 152 coliform strains were used (26 stable and 126 unstable) With each of the strains inoculations were made of 30 tubes containing dextrose phosphate medium They were divided into six sets of five tubes each and were incubated at the six aforementioned temperatures between 22° and 37° C During the following five days one tube was taken daily and examined for MR reaction

CHART 1

Different Types of MR Reactions

Day of incubation	Incubation temperature					
	22°	27°	30°	33°	35°	37°
1		±	+	±	+	+
2	+	+	+	+	+	+
3	+	+	+	+	+	+
4	+	+	+	+	+	+
5	+	+	+	+	+	+

Stable MR positive strains

Day of incubation	Incubation temperature					
	22°	27°	30°	33°	35°	37°
1		−	±	−	±	±
2		−	−	−	−	−
3		−			−	−
4			−	−		
5		−				−

Stable MR negative strains

Day of incubation	Incubation temperature					
	22°	27°	30°	33°	35°	37°
1	−	±		±	+	±
2				+	+	+
3				+	+	+
4				+	+	+
5				+	+	+

Unstable strains group 1

Day of incubation	Incubation temperature					
	22°	27°	30°	33°	35°	37°
1		±	±	+	±	+
2	−	−	+	+	+	+
3	−	−	−	±	+	+
4		−	−	−	±	+
5	−		−		−	+

Unstable strains group 2

After 24 hours of incubation the MR reactions were irregular After 48 hours the stable strains all showed clearly positive or clearly negative reactions at all incubation temperatures and the reactions did not change under continued incubation up to the *fifth day*

The unstable strains, on the other hand, showed differing reactions after 48 hours and longer time of incubation at the different temperatures These strains could be divided into two groups (Chart 1) In one group (1) the temperature threshold did not change during five days

TABLE 3

Unstable Strains

Type	IMVC	Number of strains able to produce gas up to										Total	Grand total	% of all 126 unstable strains
		22°	27°	30°	33°	35°	37°	39°	41°	43°	45°			
Interm	+ — + —	–	–	10	–	–	1	–	–	–	–	11	20	15.9 %
	— — + —	–	–	2	2	1	4	–	–	–	–	9		
B aerog	+ — + +	1	2	–	1	2	5	10	8	5	–	34	106	84.1 %
	— — + +	–	1	1	1	15	36	15	2	–	1	72		
Total		1	3	13	4	18	46	25	10	5	1	126	126	100 0 %

yielded positive reactions between 22° and 37° C The stable strains, on the contrary, yielded uniform reactions on all occasions

As seen from Table 3, 84 1 per cent of unstable strains were B aerogenes and 15 9 per cent intermediaries Among 224 strains capable of producing gas up to 45° C, only one was unstable (0 4 per cent) Of 389 strains which produced gas only at temperatures below 45° C, 125 were unstable (32 per cent)

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CHART 1

Different Types of MR Reactions

Day of incubation	Incubation temperature 22	27°	30°	33°	35°	37°
1		+	+	±	+	+
2	+	+	+	+	+	+
3	+	+	+	+	+	+
4	+	+	+	+	+	+
5	+	+	+	+	+	+

Stable MR positive strains

Day of incubation	Incubation temperature 22	27°	30°	33°	35°	37°
1		–	±		±	±
2		–	–	–		–
3	–					–
4		–				–
5			–		–	

Stable MR negative strains

Day of incubation	Incubation temperature 22	27	30	33	35	37°
1	+	+		+	+	±
2				–	+	+
3				+	+	+
4				+	+	+
5				+	+	+

Unstable strains group 1

Day of incubation	Incubation temperature 22°	27°	30°	33°	35°	37°
1		±	±	+	±	+
2	–	–	+	+	+	+
3	–	–	–	±	+	+
4		–	–	–	±	+
5	–		–		–	+

Unstable strains group 2

After 24 hours of incubation the MR reactions were irregular After 48 hours the stable strains all showed clearly positive or clearly negative reactions at all incubation temperatures and the reactions did not change under continued incubation up to the fifth day

aft ... differing reactions
tur ... different tempera
gr ... (Chart 1) In one
... during five days

of observation Strains were observed which showed negative reactions at 27° but positive at 30° C and above during five days of incubation, there were strains for which the threshold was 33° and others with threshold 35° C In the second group (2) the threshold temperature varied with incubation period The lower the incubation temperature, the earlier did the strain show a negative MR reaction The threshold varied in the case of several strains in group 2 It could extend from 35° on the second day to 37° on the third, from 22° on the second day to 30° on the fifth, and so on

It is evident that 48 hours of incubation was sufficient for stable strains and for unstable group 1 strains, but insufficient for unstable group 2

TABLE 4
89 Unstable Coliform Strains

	Number of negative MR reaction					
Incubation at	22°	27°	30°	33°	35°	37°
After 1 day	53	17	9	1		–
After 2 days	70	71	62	38	13	–
After 3 days	88	87	73	47	17	4
After 4 days	88	88	79	51	20	13
After 5 days	89	88	81	56	23	15

For determination of incubation period the following experiments were also conducted Eighty-nine unstable coliform strains were incubated at six different temperatures for five days MR reactions were recorded daily The results are shown in Table 4 It will be seen that incubation at 22° C afforded the largest number of negative reactions after five days of incubation Incubation at 22° C however, produced an uncertain result on another occasion when three strains showed intermediary (±) reactions instead of the negative reactions obtained at 27° C and higher temperatures With incubation at 27° C there were 87 negative reactions after only three days of incubation, but only 81 negative after five days of incubation at 30° C Incubation at 33°, 35° and 37° C produced a considerably smaller number of negative reactions

Relationship between pH and MR The following experiments were carried out to study the relationship between the pH value in the cultures and the MR reaction An electrical pH meter was used for this purpose

To cultures of eight MR positive and seven MR negative coliform strains 0 04 per cent of ordinary methyl red solution and 0 04 per cent of sodium methyl red solution respectively, were added to check the effect on the pH value The measurements revealed no changes and

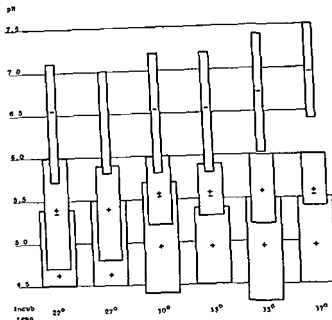


Fig 1
Distribution of pH values of pos ± and neg MR reactions at incubation temperatures of 22 37° C during 5 days 33 coliform strains

thereafter the pH values were therefore determined after reading of the MR reaction

For study of the pH value in cultures of coliform strains use was made of 7 laboratory strains, 45 strains from faeces and 55 strains from water pH was determined and MR reaction read on 2541 different cultures

Fig 1 shows the distribution of the pH values for negative, ± and positive reactions of 33 coliform strains at different incubation temperatures The pH ranged from 4 3 to 5 7 for positive, 4 7 to 6 0 for ±, and 5 7 to 7 5 for negative reactions The figure also shows that the pH value for negative reactions tended to increase with higher incuba tion temperatures For intermediary (±) reactions the distribution of the pH values decreased with higher incubation temperatures For positive reaction all six incubation temperatures produced almost the same range of value (pH 4 5 5 5) The results are in agreement with those of *Clark & Lubs* (1917 a) The latter found the colour change zone of the indicator to be in the pH range 4 4–6 0

For negative reactions the pH values *rose* from the second to the fifth day of incubation from on an average, about 6 0 to 6 6 6 8 and the yellow colour of the negative MR reaction became paler For positive reactions the pH values *fell* on an average, from about 5 0 to 4 6–4 7 during five days of incubation and the red colour became darker at all

incubation temperatures. There were transitional brown ± reactions which after lengthy incubation usually had a yellow shade. Among 613 strains examined two from water were found which at all temperatures yielded ± reactions after three days of incubation. The shades of the MR reactions did not however completely agree with the pH values of all coliform strains. There were pale yellow reactions which produced at pH of only 5.7–6.0 and dark yellow reactions with pH 6.3–6.9. Dark red positive reactions usually showed pH 4.6–4.8 but *sometimes pH 5.2–5.4. Therefore a division of MR reactions into more* than three shades as proposed by *Stuart et al.* (1938) is hardly of value. The MR reaction should be read within 15 minutes since after that time the colour of the indicator changes.

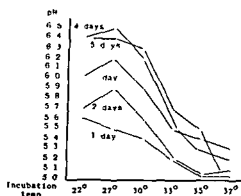


Fig. 2
Relation of pH and MR. Average of pH values of 13 unstable coliform strains at *incubation temperatures of 22–37° C during days*

Incubation temperature and pH value. As shown by Fig. 1 incubation at 22° C must be considered unsuitable since ± reactions covered a wide range which makes the assessment difficult. Incubation at 27° yielded more distinct results which improved still further at higher *temperatures. Only in the case of unstable strains do incubation tem*perature and incubation time have a decisive effect on the MR reaction. Thirteen unstable strains were therefore examined and their mean pH value was determined. The results are shown in Fig. 2. Since positive MR reactions give a pH of max. 5.7 and ± reactions max. 6.0 two days of incubation *is not sufficient to distinguish negative from ±* reactions of unstable coliform strains. Not until the third day did the pH rise to 6.2 but only at 27° C. The required level had thereby been attained and a longer period of incubation was considered unnecessary. At 30° C pH 6.2 was not observed until the fourth day and never at higher temperatures.

The pH measurements thus showed that the incubation temperature should be *27° C and the incubation period not less than 3 days.*

Different kinds of peptone. Clark & Lubs (1917 b) reported that accurate determination of the MR reaction was not possible with other

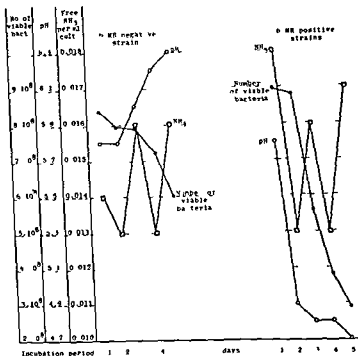


Fig 3
Free NH_3 from coliform strains (average

than Witte's peptone The following six kinds of peptone were therefore tried in dextrose phosphate medium (1) Wilson & Co Chicago (2) Witte (3) Parke Davis & Co bacteriological peptone (4) Protesosepeptone Difco (B 120) (5) Schmitt Jourdan "indole free peptone" (6) Orthana peptone With all kinds of peptone dextrose phosphate media showed pH 7 9 8 0 before and pH 7 5 after sterilization The investigation comprised 10 coliform strains from faeces 125 coliform from well waters and 40 gram negative rods which did not produce gas from lactose and which were isolated from well waters Each strain was inoculated into 6 dextrose phosphate tubes containing different peptones and incubated at 27° C for three days Out of 175 strains examined 118 showed identical reactions with all kinds of dextrose phosphate media whereas 57 strains (32 5 per cent) gave different reactions with different kinds of peptone Orthana peptone yielded 25 more positive reactions than the remaining peptones between which the differences were negligible Orthana peptone was therefore unsuited for the MR test In the present study use has been made of Wilson & Co peptone

Effect of ammonia on MR reaction There has been discussion in the literature as to whether formation of ammonia in the cultures has any

effect on the MR reaction *Clark & Lubs* (1915) *originally considered this possible but later* (1917 b) declared that the quantity of ammonia formed in the cultures is too small to have any effect The question of the role of ammonia formation in the MR reaction has not yet been unambiguously answered It therefore appeared a matter of interest to determine the quantity of ammonia in cultures of coliform strains in connection with the MR test For this purpose 11 MR positive and 11 MR negative strains were studied *in respect to pH value, number of* living bacteria and quantity of NH_3 Samples were collected for examination *daily during five days of incubation at 27° C from a dextrose* phosphate culture The quantity of NH_3 was determined by a micro-Kjeldahl method modified by *Mirsky* (1936)

Fig 3 shows the mean values of number of living *bacteria, pH and quantity of* NH_3 per ml of culture obtained with six negative and six positive strains during five days of incubation The number of living bacteria fell after the second day both for negative and positive strains *while the pH value rose every day in negative and fell in positive* strains The quantity of NH_3 was irregular and followed neither the number of living bacteria nor the pH value

The quantity of free NH_3 was determined in 100 dextrose phosphate cultures No difference was found between MR positive and MR negative strains In both cases the quantity of NH_3 was 0 011 0 028 mg per ml of culture in 90 per cent of the strains

The quantity of NH_3 *varied between* 0 005 and *0 024 mg per ml in* MR negative and between 0 008 and 0 028 mg per ml in MR positive strains, *i e* the quantities and the variations were alike in positive and negative strains

To check these results the quantity of free NH_3 of five different kinds of peptone was determined The peptone was dissolved in distilled water and sterilized in the same way as for *preparation of dextrose* phosphate medium *The results are listed below*

Wilson & Co Chicago (the kind used for this study)	0 0326 mg NH_3/ml
Witte	0 0372
Bacto tryptone	0 0318
Orthana	0 0292
Indole free Schmitt Jourdan	0 0348

The quantity of NH_3 was thus rather larger in the peptone than in the dextrose *phosphate cultures Accordingly ammonia* was *present* before the cultivation in peptone and was not formed in the culture as a product of metabolism of the bacteria

To find out which quantity of ammonia would affect the MR reaction, the following test was carried out Sterile dextrose phosphate medium was adjusted to pH 4 5 and produced a strong positive MR reaction at that level To attain pH 6 0 with a negative MR reaction, 0 094 mg of NH_3 per ml must be added To convert a moderately positive MR reaction of pH 4 8 to a negative reaction of pH 6 0, 0 072 mg of NH_3 per

ml must be added to the medium To attain the same level as the medium at pH 5 15 which produced a faintly positive reaction 0 052 mg of NH_3 per ml must be added The quantity of ammonia present in dextrose phosphate cultures was thus not sufficient to convert a positive MR reaction to a negative

DISCUSSION

A uniform method must be used for typing coliform strains since variations in the technique produce different results in typing The most important factors in performance of the MR test are temperature and time incubation of the bacteria *Clark & Lubs* who proposed the methyl red test in 1915 started The incubation period may seem long to those impatient for results but we urge a five day period at 30 C with no apology Nevertheless incubation at 37° C came very soon to be used In 1916 *Levine* gave it as his opinion that a two day incubation period at 37° C was sufficient for the MR reaction Several authors have maintained that dextrose phosphate cultures should be incubated at 30° C (*inter alia Chen Chong Chen et al* 1920 *Henriksen* 1955 *Koser* 1926 *Levine* 1941 *Stand Meth* 1955 *Stuart et al* 1940 *Vaugh et al* 1939 *WHO* 1958) but at temperature of 37° C is still widely employed

Since the characteristics of the strains one wishes to study are not known in advance the technique should be worked out with the consideration of strains apt to give discrepant results and requiring a low incubation temperature and a long incubation period The present investigations show that incubation at 27° C produces a larger number of MR negative strains than at 30° C and that the incubation period can be shortened from five to three days A short incubation period is desirable for routine water tests and is very important in bacteriological studies of coliforms for clinical purposes *Gale* (1946) states that the production of amino acid decarboxylases by strains of E coli is inhibited by growth temperatures of the order of 37° C and that optimum formation of the enzymes takes place when growth occurs at 20° 26° The larger number of MR negative strains at 27° than at 30° may therefore be explained by the fact that enzymes which decarboxylate amino acids during the growth of coli develop best at 20° 26° C

The investigations of the pH values confirmed that incubation at 27° gives better results than at 30° C

Of the six qualities of peptone studied five appeared to be equivalent while one proved unsuitable for the MR test The difference of reaction with different kinds of peptone reduces the reliability of the MR test since the method of investigation cannot be standardized Variations may also occur between different batches of the same kind of peptone

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Clark & Lubs (1915) *Topley & Wilson* (1957) and others assume that ammonia is formed by coliform bacteria though in insignificant

quantities The present studies, however, show that no increase in ammonia content takes place when coliform bacteria are grown in dextrose phosphate medium The ammonia content in the cultures was in fact less than in uninoculated peptone water

SUMMARY

Different species of coliform bacteria yield different MR reactions at different temperatures and periods of incubation Incubation of dextrose phosphate culture at 27° C is recommended since at this temperature the incubation period can be limited to three days instead of at least five days at 30° C

The choice of peptone quality has an important effect on the MR reaction Of six qualities tested, five appeared to be equivalent

The content of ammonia in a culture does not increase during growth of coliform bacteria, and ammonia formation has therefore no significance for the MR reaction

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THE VETERINARY INSTITUTE OSLO NORWAY (DIRECTOR DR O GRINI)

KERATINOMYCES AJELLOI AND MICROSPORUM COOKEI IN NORWEGIAN SOIL

By

KÅRE LINDQVIST

Received 11 x 60

Soil constitutes the main reservoir for a number of fungi pathogenic to man and animals (3, 12) The dermatophytes are the most widely distributed pathogenic fungi and the increasing frequency with which they are found in soil becomes an important factor in the epidemiological and epizootiological studies of dermatomycosis The world wide distribution and infectious character of dermatomycosis have prompted the Joint WHO FAO Expert Committee on Zoonoses to strongly recommending further survey and research in all fields of human and animal dermatomycoses (5)

Several reports have appeared on the presence of dermatophytes in soil special attention having been paid to *Microsporum gypseum* (2, 3, 11 12 14 22) Another well known pathogenic species *Trichophyton mentagrophytes*, has also been isolated from soil (19) With the recent recognition of *Keratinomyces ajelloi* as a dermatophyte (13, 15, 24), this species must now be regarded as the most common dermatophyte in soil as is evident from the reports of its presence in most parts of the world (15) When describing *K ajelloi*, *Vanbreuseghem* referred to the fungus as a dermatophyte although its pathogenicity was not proved at that time (26)

In 1959 three reports appeared which demonstrated the pathogenicity of *K ajelloi* *Georg et al* recovered the fungus from ringworm lesions in a squirrel (15) They were able to prove the pathogenicity of this strain for guinea pigs *Rieth & El Fiki* described a case of dermatomycosis in a horse, which was definitely caused by *K ajelloi* (24) Noteworthy is especially the microscopic appearance of the fungus in the infected hairs from this horse *Evolceanu & Alteras* isolated four strains of *K ajelloi* from soil (13) One of the strains proved to be pathogenic for mice, guinea pigs and man The authors proposed the name *Epidermophyton terrigenum*, a suggestion which will be returned to later in this paper

Soil may also harbour non-pathogenic fungi which may be mistaken for dermatophytes Such a species is *Microsporum cookei* *M cookei* was first isolated by *Cooke*, who regarded the fungus as a variety

of *M gypseum* (8) The fungus has since been isolated on several occasions from rodents (21), from large wild animals (20), and from soil (22) The animals from which *M cookei* was isolated, presented no symptoms of dermatomycosis The fungus is described in the cited papers as a red variety of *Microsporum Ajello* in 1959 recognized it as a separate species and named it *Microsporum cookei* (1)

A mycologist working with dermatomycosis of animals is faced with a special problem because the heavy haircoat of animals is frequently contaminated with dust and soil rendering mycological examination difficult due to the growth of a number of species of no pathological significance The introduction of selective media greatly facilitated the isolation of pathogenic fungi from contaminated material Some non-pathogenic soil fungi, however, will grow on these media and a knowledge of species liable to causing confusion with the dermatophytes becomes a necessity Representative species of this kind are *M cookei* and *Trichophyton terrestre* (10)

A common method for the isolation of keratinophilic fungi from soil is the "hair bait technique" originally developed by *Karling* in 1946 for the isolation of keratinophilic chytrids from soil and water (17), and later, in 1952, adapted by *Vanbreuseghem* for the isolation of dermatophytes from soil (25, 26)

During a routine search for dermatophytes in Norwegian soil using the hair bait technique, several strains of *K ajelloi* and three strains of *M cookei* were encountered, a presentation of which is given below

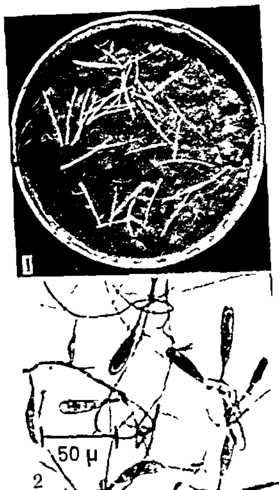
MATERIAL AND METHODS

59 soil samples obtained in the Oslo area were subjected to the hair bait technique Microscopical examination was performed as soon as growth of fungi was observed For pure culture studies the hairs were inoculated on Mycobiotic Agar (Difco) and Sabouraud's glucose agar Incubation was carried out at 25° C Microscopical examinations of the cultures were performed using cellophane tape mounts of the specimens This method consists of using a piece of transparent adhesive tape part of which is allowed to touch the fungus colony … to apply some pressure to the tape during sampling … blue is placed on a slide and the tape is streched … elements attached to the tape come in touch with … stretching the tape now adhering to the slide thin preparations can be made where the fungus elements remain in their proper arrangements Provided a good quality tape is used the preparations are very well suited for micro photography

The microphotographs of dermatophytes are all taken from recent isolates in the authors *collection*

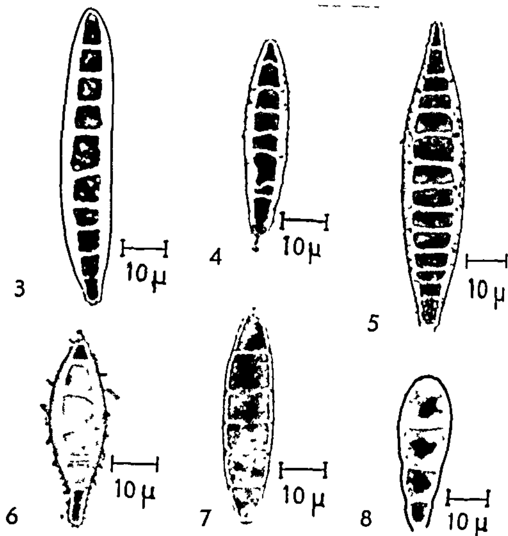
OBSERVATIONS

Keratinomyces ajelloi 43 of the 59 soil samples yielded growth of *K ajelloi* 42 of the positive soils originated from cultivated areas 16 samples were negative for keratinophilic fungi 14 of which were from non-cultivated soil On the baited soils *K ajelloi* appeared after 2 3 weeks incubation as light brownish layers of macroconidia and hyphae covering the hairs (Fig 1) The mature macroconidia of this fungus



Figs 1 and 2
Fig 1 The hair bait technique *Fig 2* Macroconidia from greyish part of colony of K ajelloi

have thick, smooth walls in contrast to the rough-walled macroconidia of *M canis*, *M equinum*, *M gypseum* and *M cookei* (Fig 3) The lengths of the macroconidia were about 40 60 μ Macroconidia of all the above species may in the early stages of development appear almost identical On Sabouraud's agar *K ajelloi* sometimes developed sections in the colony with a greyish colour These sections consisted of hyphae bearing macroconidia which seemed to be poorly developed (Fig 2) In some strains microconidia were also observed The normal colour of the colony was varying shades of brown Some strains produced a dark brown to almost black pigmentation on the reverse side of the colony The entire agar may become ochraceous brown Most strains en-



Figs 3 8

Fig 3 Macroconidium of Keratinomyces ajelloi *Fig* 5 Macroconidium of Micro- *Fig* 6 Macroconidium osporum gypseum — ccosum

countered in this material, however, have been devoid of this pigmentation but have in other respects conformed well with the original description of the species (26) Cultures of *K ajelloi* exhibited a characteristic odor and developed well with an abundant production of macroconidia on hairs in distilled water

Microsporum cookei Two soil samples originating from flowerbeds heavily fertilized with animal manure and one sample from a barn yard yielded growth of *M cookei* as well as *K ajelloi* and a third keratinophilic species *M cookei* developed a powdery colony resembling that of *M gypseum* However, a marked bloodred pigmentation was noted on the reverse side of the colony This pigmentation was soon lost upon subculturing Microscopically, the fungus was found to

produce numerous large thickwalled and multiseptate macroconidia (Fig 4) Clavate microconidia were also observed Some of the macroconidia with their rough walls were almost indistinguishable from those of *M canis* (Fig 5) Another species *M equinum* which is closely related to *M canis* may also produce macroconidia of the type observed in *M cookei* (Fig 6) The thin walled macroconidia of *M gypseum* presented no great problem in differentiation (Fig 7) *M cookei* grew well on sterile hairs in distilled water The cultural and microscopical characteristics were in good agreement with Ajello s description of the species (1)

Some soil samples yielded growth of other keratinophilic fungi which did not present any problem in the differentiation from the dermatophytes and therefore will not be described here *M gypseum* was not found

DISCUSSION

Before the discovery of the pathogenicity of *Keratinomyces ajelloi* the finding of this fungus represented only a minor problem in the differentiation from the common dermatophytes Its presence in dermatomycotic lesions was regarded as purely due to contamination Although only a minority of the isolates from soil may prove to possess the power of invading the keratinized tissues of the living animal *K ajelloi* has become a most important member of the dermatophyte group because of its common occurrence in soils in various parts of the world The results recorded in this paper show that *K ajelloi* is the predominating species of the keratinophilic flora of Norwegian soil It is evident that it is more common in cultivated that non cultivated soil The various strains have shown some variation in characters Some cultures may have a powdery light to darker brown surface with or without a darkbrown or ochraceous pigmentation on the reverse side Non pigmented strains were also described by *Ajello* in the United States (2) Microconidia are usually not observed but their presence has been definitely established by *Georg et al* (15) Microconidia were also observed by *Rieth & El Fiki* in their isolate of *K ajelloi* from dermatomycosis in a horse caused by this fungus (24)

[illegible] icroconidia
er [illegible] *et al* have
re [illegible] (15) in their re description
Georg et al maintain the genus *Keratinomyces* and the species designation *K ajelloi* as proposed by *Vanbreuseghem* (26) *Fvolceanu & Alteras* on the other hand propose the name *Epidermophyton terrigenum* (13) for the following reasons microscopical aspect and inability to invade the hairs in the infected animal The specific epithet *terrigenum* was given because of the terrestrial origin of the fungus There seems to be very little reason to follow their suggestion of a new name *Epidermophyton floccosum* the hitherto only species within

this genus produce thin-walled and usually club-shaped macroconidia which differ markedly from those commonly found in *K ajelloi* (Fig 8) The ability to invade the hairs in the infected host has been shown beyond doubt by *Rieth & El-Fiki* in their nice preparation of an infected hair from a horse (24), and by *Georg et al* who found hyphae within the hairs of both their spontaneous case in a squirrel and in the experimentally infected guinea pigs (15) The latter paper also contains microphotographs of microconidia, which were also observed by *Rieth & El-Fiki* and the present author Accordingly, the criteria of *Evolceanu & Alteras* for assigning *K ajelloi* to the genus *Epidermophyton* are not present There seems to be much stronger reasons for an inclusion of *K ajelloi* in the genus *Microsporum* For the time being, however, it is probably better to preserve the name originally proposed for this microorganism

The discovery of *Dawson & Gentles* of a prefect stage of *K ajelloi* may warrant another re description of the species (9) Their important finding of cleistothecia and asci containing eight bright yellow asco spores with finely echinulate walls places *K ajelloi* within the Ascomycetes There is no doubt as to origin of the cleistothecia as *K ajelloi* was obtained by culture of single ascospores

The perfect stages of dermatophytes have been difficult to find and most mycologists place these fungi in the Fungi imperfecti *Nannizzi*, on the other hand, pointed out the great similarities that exist between the dermatophytes and the members of the perfect family Gymnoascaceae (23) Further research along these lines are needed to establish the correct taxonomic position of the dermatophytes

The closely septate hyphae observed in infected hairs and scales seem very characteristic of *K ajelloi* and it is possible that one may arrive at a correct diagnosis by direct microscopical examination alone Although this way of arriving at a species diagnosis may not prove correct in every case of *K ajelloi* infection, it may discover most cases as doses the direct microscopical examination of hairs from infections caused by *T verrucosum* (18) *K ajelloi*, however, grows well on the ordinary media and is not inhibited by penicillin, streptomycin, chloramphenicol or cycloheximide (Actidione) in concentrations commonly added to selective media for pathogenic fungi The cultural determination of *K ajelloi* is easy and should of course always be carried out

Three strains of *M cookei* were found in this small survey The pathogenicity of this fungus has as yet not been proved and further research is necessary to determine wether or not *M cookei* should be called a dermatophyte In spite of the fact that it has been isolated on several occasions from hair samples from animals, its occurrence in such material must be regarded with caution Culturally, *M cookei* may resemble some strains of *M gypseum*, but microscopical examination will reveal the differences between these two species *M cookei*

will produce both macro and microconidia which may be very similar to those produced by *M canis* and *M equinum*, while the thin walled macroconidia of *M gypseum* can be easily distinguished The mean length of the macroconidia of *M cookei* is about 50 μ, while of *M canis* 74 μ according to *Conant* (7) *M audouini* does not represent a diagnostic problem as it differs markedly from the other *Microsporum* species

The cultural appearance on Sabouraud agar with the development of a deep red pigment will also distinguish *M cookei* from *M canis* and *M equinum* *M canis* develops a white aerial mycelium and a bright yellow pigment in the agar *M equinum* on the other hand, grows more slowly produces little aerial mycelium and shows a brownish pigmentation of the colony Only a very limited number of macroconidia is produced in primary cultures of *M equinum* on Sabouraud s medium in contrast to the abundant mass usually observed in cultures of *M cookei* *K ajelloi*, *M gypseum* and *M canis*

Macroconidia of dermatophytes are never produced in the infected animal and are only observed when the fungi lead a saprophytic existence The finding of macroconidia of *M gypseum* in a soil sample by *Gordon et al* furnished the definite proof of the saprophytic existence of this species in soil (16)

Observations have been published however, on the presence of macroconidia in *M canis* infections (6) According to *Ajello*, these spindle shaped structures have actually been cells from the root sheath cuticle (4)

SUMMARY

A small survey has been made to determine the presence of *Keratinomyces ajelloi* in Norwegian soil 59 soil samples were examined by the "hair bait technique 43 yielded growth of *K ajelloi*

Microsporum cookei was found in three samples *Microsporum gypseum* was not found The soil samples originated from the Oslo area

The present position of *K ajelloi* as a dermatophyte and the importance of the ubiquitous occurrence of this fungus in soil are discussed

Characteristic features of cultures of *K ajelloi* and *M cookei* as compared with some common dermatophytes are stressed

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STUDIES ON THE INACTIVATION OF BACTERIAL VIRUSES BY NORMAL HUMAN SERUM

1 Kinetics of the Coli T 2 Phage Inactivation at Various Serum Concentrations[1]

By

LARS OLOF KALLINGS[2]

Received 5 xi 60

Coli T 2 r+ bacteriophages can be inactivated by fresh normal serum apparently in the absence of immune antibodies in the conventional sense The inactivating principle of serum is heat labile and the reaction markedly dependent on the temperature Complement and Mg^{++} seem to take part in the process (*Barlow et al* 1958) Whether the inactivation is brought about by normal antibodies or properdin alone or in conjunction is a matter of discussion (*Nelson* 1958 *Cowan* 1958)

The purpose of the experiments to be reported[3] is to study the nature and mechanism of phage-normal serum interaction

In the present and a subsequent paper (*Kallings* 1961) studies on the kinetics of the inactivation of T 2 r phages by human serum are presented Studies on reactivation and inhibition of the inactivating effect will be reported later

MATERIAL AND METHODS

Host organism A strain of Escherichia coli B was stored in sealed agar tubes at room temperature and for current use passed on agar slants two or three times a year The agar slant cultures were maintained at room temperature and transferred weekly

For each day's experiment 20–50 ml [illegible]

[illegible]

ag[illegible]

a[illegible]

[illegible] counting

Virus Stock suspension Bacteria from a broth culture growing at an exponential rate were mixed with an equal volume of a suspension of coli phage T 2r+4 diluted

[1] Aided by grants from the *Swedish Medical Research Council*

T[illegible] [illegible]ndberg and Miss *Birgitta Sund*

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and [illegible] ndinavian Congress of Pathology

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as the reactive mixtures In the controls the dilutions of native serum were substituted by the same dilutions of serum heated at 56° C for 30 minutes To test the serum for presence of heatstable phage inhibitors controls were set up where nutrient broth was added instead of serum

In each experiment the hemolytic capacity of the test system was checked as a
s a control for the
Half a ml hemo
added to 0 75 ml
sis was estimated

donors Pools of different sera were never used for the experiments

Precision of phage assay A series of 14 consecutive experiments performed on

[illegible] (mean of all individual counts 221)

TABLE 1

Analysis of Variance of Phage Assay

Source of variation	Degrees of freedom	Sums of squares	Mean squares	Variance ratio
Between samples	13	21 080	1621 54	3 78***
Within samples	42	18 023	429 12	
Total	55	39 103	710 96	

Mean individual plaque count 221

EXPERIMENTAL

Inactivation at Various Serum Concentrations

Equal volumes of serial 1 5 fold serum dilutions and a T 2 phage suspension containing 2×10^4 particles per ml were mixed and incubated for 2 hours in a waterbath at 37° C For each serum dilution the percentage of inactivated phages was computed and plotted against log serum concentrations

Well over 600 serum specimens from humans of different physiological and pathological conditions (*Kallings*, to be published) have been examined Fig 1 shows a typical inactivation curve with a steep and linear course round 50 per cent inactivation The values indicating the inactivation at the three lowest serum concentrations represent the means of 40 determinations The other values are based on the means of 8 determinations

in broth to a concentration of 1 phage particle per 400 cells. The mixture was agitated in a waterbath at 37° C for 5 minutes and 5 ml were transferred to Roux flasks containing 100 ml preincubated nutrient broth.

The cultures were incubated at 37° C for about 8 hours or until all visible turbidity had disappeared. The lysates were then centrifuged at low speed to remove debris, passed through glass filters (Jena G5), tested for bacterial sterility, dispensed in tubes and stored at +4° C. Final titers ranging from 1–2 × 10^{10} infective phage particles were ordinarily achieved.

For each day's experiment stock virus was diluted in buffer by ten fold steps.

Normal sera. Blood was drawn from apparently healthy members of the laboratory staff. After clotting of the blood for 2 hours at room temperature, serum was centrifuged at 2000 G for 30 minutes at +4° C. The sera were distributed in rubber stoppered tubes and stored in a CO_2 box until used.

Media. Nutrient broth. Difco nutrient broth 8 g and NaCl 5 g were dissolved in one liter distilled water. Aliquots of 100 ml were dispensed in screw capped bottles and sterilized.

Nutrient agar (*Barlow et al.* 1958). To prepare the plating agar 10.0 g of Difco Bacto agar, 13.0 g of Difco Bacto tryptone, 8.0 g sodium chloride, 2.0 g sodium citrate and 1.3 g glucose were mixed in one liter distilled water and heated in boiling water until the agar dissolved. The plating agar was divided into 500 ml aliquots.

Top layer soft agar for the assay of phage by the agar layer method consisted of 6.0 g Difco Bacto agar, 10.0 g Difco Bacto tryptose, 8.0 g sodium chloride, 2.0 g sodium citrate and 3.0 g glucose in one liter distilled water. This was divided into 300 ml aliquots.

For the preparation of agar slants 2 per cent nutrient agar was used.

All media were brought to values giving a final pH of 7.4 and sterilized on two consecutive days by steaming for 30 minutes and stored at +4° C.

For each day's experiment the plating and top layer agars were melted by steaming. The plating agar was poured into Petri dishes (9 cm), about 20 ml in each. The plates were allowed to solidify at room temperature and were dried for one hour at 37° C. The soft agar was pipetted into tubes and placed in a waterbath at 42 ± 0.3° C.

Diluents. Barbiturate buffer. 0.125 M was prepared by dissolving 4.124 g sodium 5.5 diethylbarbiturate, 5.350 g NaCl and 1.10 ml conc. HCl in distilled water to a final volume of one liter. The pH was adjusted to 7.4 ± 0.05.

To prepare Tris maleate buffer of pH 7.1 ± 0.1 and various molarities, Tris (hydroxymethyl) aminomethane (Sigma 7.9), maleic acid and NaOH were mixed in varying proportions to give the wanted molarity and were dissolved in distilled water.

Buffer solutions as well as 0.15 M saline and 0.1 M solution of $MgSO_4 \cdot 7H_2O$ were prepared using double glass distilled water and were sterilized by passing through a glass filter (Jena G 5).

Glassware. Neutral glass was used throughout. All glassware was cleaned by boiling in a solution of hexametaphosphate and sodium metasilicate, rinsed in running tap water, 0.1 per cent HCl, five times in running tap water and three times in distilled water.

Standard procedure[1] *of the inactivation test.* Serum was thawed immediately before use and diluted in the buffer.

Half a ml phage dilution containing 10^4 phage particles and 0.005 M Mg^{++} was added to the same volume of serum dilution. The reaction mixtures were incubated in a waterbath accurate to ± 0.3° C. The reaction was stopped by chilling in ice water and by 1 : 10 dilution with cold 0.15 M saline.

One ml of the dilution was pipetted into a tube containing 8.5 ml melted agar at 42° C to which 0.5 ml culture of coli B had just been added. After thorough mixing 2 ml was poured over the surface of an agar plate placed on a levelled sheet. The top layer was allowed to harden and the plate inverted and incubated at 37 ± 0.5° C overnight. On the following day the number of plaques deriving from the free phages were counted.

To determine the proportion of phages inactivated, the initial number of phage particles was assayed by plating at least four control tubes treated in the same way

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1 Mainly the same as the method described by *Barlow et al.* (1958).

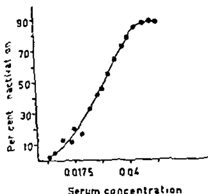


Fig 2
Inactivation of T2 by varying serum concentrations at 37° C

Abscissa Arithmet value of serum concentration on log scale
Reaction mixture T tal volume 1 ml 1 × 10⁴ T2 particles phage and serum (M L.) dil in barb turate buffer 0 125 M at pH 7 4 ± 0 05 0 00?5 M Mg⁺ Incubation time 2 hours

The slope of the curve represents the rate at which inactivation proceeds at varying serum concentrations If the rate was solely dependent on the content of inactivating principle in the serum or an expression of the resultant of antagonistic forces in a constant ratio the linear parts of the inactivation curve of different sera should run a parallel course

The equation (2) with varying values of a should thus hold for all sera within the range of logarithmic progress of inactivation The quotient $\frac{^aS_1 \quad ^aS_2}{k}$ should give the logarithmic expression of the difference in inactivating capacity between two sera S_1 and S_2

In fact the inactivation curves of most sera have been found to be roughly parallel as will be reported in a following paper

The persistent fraction Experiments were set up to investigate why the increase of serum concentration above a certain level did not influence the degree of inactivation The experiments were intended to elucidate whether there was a resistance towards serum action due to properties of the persistent phage particles themselves or whether the persistence was due to lack of cofactors presence of inhibitors or due to inherent qualities of the test procedure such as reactivation due to dilution

Firstly tests were made to see if the resistance towards serum action was a stable property of the phage particle transferable to the progeny Isolated plaques were picked up from platings of reaction mixtures showing 9a per cent inactivation The plaques were transferred to broth incubated centrifuged filtered and titrated as described for stock virus The inactivation of the resulting virus suspensions each

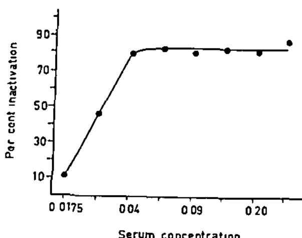


Fig 1

Inactivation of T2 by varying serum concentrations at 37° C

Abscissa Arithmet value of serum concentration on log scale
Reaction mixture Total volume 1 ml 1×10^4 T2 particles phage and serum (A A) *dil in barbiturate buffer 0 125 M at pH 7 4 ± 0 05 0 0025 M* Mg^{+} *Incubation* time 2 hours

As is seen in the fig, inactivation does not reach 100 per cent in spite of increasing serum concentration It appeared that after a reaction time of 1–2 hours at 37° C, none of the sera examined had inactivated all or nearly all phage particles A persistent fraction of 5–25 per cent of initial phage activity regularly remained

The close dilution steps of the standard inactivation test are obviously too great to admit a detailed study of the steep part of the curve and the slope at the intercept on the abscissa Fig 2 demonstrates the inactivation curve of another serum specimen diluted so as to give smaller *differences between the serum concentrations* This curve proved to be sigmoid with a linear midpiece between 25 and 75 per cent inactivation

The deviation from the straight line at decreasing serum concentration was *found to start between 10 and 30 per cent inactivation*, varying with different sera

Under the experimental conditions described the logarithmic course of inactivation may be described as

$$(1)\quad \frac{p_0 - p}{p_0} = k \log c - a$$

in which p_0 = the initial number of phage particles p = the number of phage particles remaining *active at the end of the incubation period* c = serum concentration and k and a are constants

Satisfied by the experimental values corresponding to the three lowest serum concentrations in Fig 1 the equation will run

$$(2)\quad y = 1\ 96 \log x + 3\ 56$$

The slope of the linear part of the curve in Fig 2 gives k = 1 87

TABLF 2

The Effect of the Addition of Serum and Further Incubation on Persisting Phage Activity

	Mixtures*				
	P_0	P_1	P_2	P_3	P_k
Number of phage particles/ml × 10^2	129 3	44 3	29 8	2×8 5	2×13 7
Residual activity in per cent		34	23	13	21
Per cent inactivation of the activity persisting after 1 hour at 37° C		–	33	62	38

* Interpretation of the symbols will be found in the text

It appears that continued incubation as well as the addition of fresh serum diminishes the persistent fraction In all experiments the prolonged incubation in a second reaction mixture (P_k = serum dilution 1 10) increased the inactivation more than continued incubation of the primary reaction mixture (P_2 = serum dilution 1 5) for the same length of time

It was considered necessary to test if the persistent fraction was resistant to any concentration of serum in the primary reaction mixture Increasing volumes of serum were therefore added to a series of tubes containing a constant number of phages The mixtures were brought to a constant total volume with barbiturate buffer and treated according to the standard procedure Four different sera were tested in six experiments The result of two experiments is given in Fig 4 After a reaction time of one or two hours the inactivation was found to proceed very slowly or to be arrested within a broad range of in-

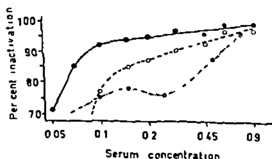


Fig 4

Inactivation of persistent T2 by high serum concentrations at 37° C

● ●—● Serum 9839 2 hours incubation

Serum 9839 1 hour incubation

○ ○ ○ Serum (1 L.) 1 hour incubation

Abscissa Arithmet value of serum concentration on log scale

Reaction mixture Total volume 1 ml 1 × 10^4 T2 particles phage and serum dil in barbiturate buffer 0 125 M at pH 7 4 ± 0 05 0 0025 M Mg

deriving from a single plaque, was tested at various serum concentrations As shown in Fig 3 there was only a slight difference between the inactivation of stock virus and virus propagated from the persistent fraction The small difference in reaction rate actually found may be attributed to the different age of the two phage lysates involved (cf Fig 9).

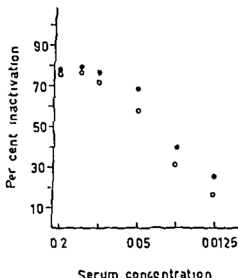


Fig 3

Inactivation of stock phages and phages propagated from the persistent fraction by varying serum concentrations at 37° C

● Stock phages ○ Phages propagated from a plaque produced by a serum survivor
Abscissa Arithmet value of serum concentration on log scale
Reaction mixture Total volume 1 ml 1×10^{4} T2 particles phage and serum (1 1) in barbiturate buffer 0 125 M at pH 7 4 ± 0 05 0 0025 M Mg^{++} Incubation time 2 hours

In another series of experiments the persistent fraction was incubated with additional fresh serum

Using cold barbiturate buffer as diluent, a mixture containing 0 2 ml fresh serum and about 1×10^{4} phage particles per ml was prepared The mixture was divided into one ml aliquots in tubes and incubated in a waterbath at 37° C for periods of one and two hours After the incubation, four tubes from each period of incubation (P_1, P_2) were chilled, the mixtures diluted and assayed for phage activity as described above From other tubes incubated for one hour, 0 5 ml aliquots were added to eight tubes, four (P_s) containing 0 2 ml fresh serum and 0 3 ml barbiturate buffer and four (P_k) containing 0 5 ml nutrient broth The tubes were incubated for one hour at 37° C, chilled, the mixtures diluted and titrated for residual phage activity To determine the initial number of infective phage particles (P_0), eight tubes containing 0 5 ml of the phage dilution and 0 5 ml nutrient broth were included in the experiment The result is summarized in Table 2 The experiment was repeated using two other sera with consistent results

TABLE 2

The Effect of the Addition of Serum and Further Incubation on Persisting Phage Activity

	Mixtures*				
	P_0	P_1	P_2	P_s	P_k
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Per cent inactivation of the activity persisting after 1 hour at 37° C		-	33	62	38

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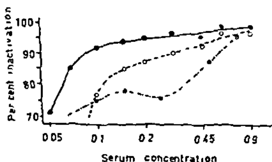


Fig 4

Inactivation of persistent T2 by high serum concentrations at 37° C

●—●—● Serum 9839 2 hours incubation
Serum 9839 1 hour incubation
○ ○ ○ Serum (I L) 1 hour incubation
Abscissa Arithmet value of serum concentration on log scale
Reaction mixture Total volume 1 ml 1 × 10^4 T2 particles phage and serum dil in barbiturate buffer 0 125 M at pH 7 4 ± 0 05 0 0025 M Mg

creasing serum concentration Above a certain level of serum concentration the inactivation effect of two of the sera tested slightly decreased as illustrated by the course of the bottom curve in Fig 4 With all sera the inactivation achieved a maximum level of 95 per cent or more The phenomenon of an intermediary maximum point on the inactivation curve was occasionally observed in other experiments with different sera

As also shown in the figure a doubling of the reaction time displaced the curve to higher inactivation values but did not essentially change its course

Inactivation at Various Reaction Times and Serum Concentrations

In a series of experiments, equal amounts of a phage dilution containing about 2×10^4 particles/ml and a given serum dilution were mixed and divided into aliquots of 1 ml These were incubated for varying periods at 37° C and tested for phage inactivation In order to correct for the "spontaneous" phage inactivation during the incubation, controls with dilutions of the phages and nutrient broth or serum previously inactivated at 56° C for 30 minutes were included in the experiments The phage inactivation attributable to the serum effect was calculated on the basis of the controls plated after each time of incubation As shown in Fig 5 the inactivation continuously increased with time up to 90–95 per cent inactivation and then remained fairly constant With the serum and serum concentration used in this experiment 99 100 per cent inactivation was reached only after an incubation period of 18 hours

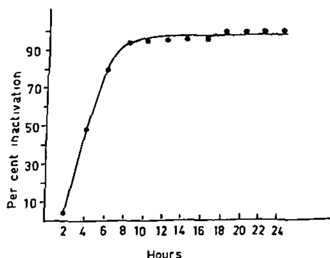


Fig 5

Inactivation of T2 by normal serum dil 1 85 at varying reaction times and 37° C

Reaction mixture Total volume 1 ml 1×10^4 T2 particles phage and serum (VA) dil in barbiturate buffer 0 125 M at pH 7 4 ± 0 05 0 0025 M Mg⁺

To study the relation between the rate of inactivation and the concentration of serum, constant amounts of phage dilution were added to identical series of serial serum dilutions. The series were incubated for varying periods and tested for phage inactivation. In this way 10 different sera were tested in 35 experiments.

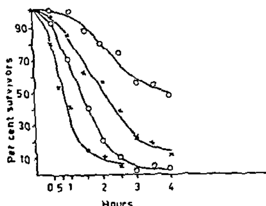


Fig 6

Time survivor curves of T2 at various concentrations of normal serum

[illegible] — 7 1 25 1 38 and 1 57
[illegible] T2 particles phage and serum (M L)
7 4 ± 0 05 0 0025 M Mg^{++} Temperature

Fig 6 gives the shape of the time survivor curves at various concentrations of a serum lot. As will be seen the rate of inactivation depends on the serum concentration. There is an initial period of acceleration, a phase of approximately steady rate and a final retardation. To check the phase of constant rate indicated the inactivation was followed at intervals of 5 minutes. All results of these tests were found to agree with a linear course of inactivation. The shape of the initial part of the curve was dependent on the dilution of serum. At the lower serum concentrations tested a definite lag period could be demonstrated as shown in Fig 6 (serum dil 1 57).

The dilution giving 50 per cent inactivation was calculated from the inactivation serum concentration curve at each time of incubation (Fig 7). The reciprocals of these dilutions were plotted against the logarithms of the corresponding periods of incubation to visualize the influence of concentration upon the rate of the reaction (Fig 8). With all sera tested the rate seemed to be directly proport[illegible]

[illegible] good approach to a logarithmic relation between rate and low serum concentrations

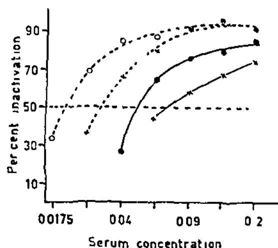


Fig 7

Inactivation of T2 by different concentrations of normal serum at various reaction times and 37° C

Abscissa Arithmet values of serum concentration on log scale Incubation time ×—×—× ½ hour, ●—●—● 1 hour, ×--×--× 2 hours, ○--○--○ 4 hours Reaction mixture Total volume 1 ml, 1×10^4 T2 particles, phage and serum (H) dil in barbiturate buffer, 0 125 M, at pH 7 4 ± 0 05 0 0025 M Mg^{++}

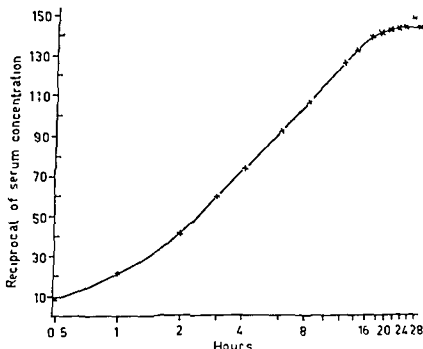


Fig 8

Inactivation of T2 by different normal serum concentrations at various reaction times and 37° C

Abscissa Arithmet values of reaction time on log scale Ordinate Reciprocal of serum concentrations inactivating 50 per cent of the initial phage activity Reaction mixture Total volume 1 ml 1×10^4 T2 particles phage and serum (A A) dil in barbiturate buffer, 0 125 M at pH 7 4 ± 0 05 0 0025 M Mg^{++}

At limiting dilutions of the sera tested, no further inactivation could be obtained by incubation periods exceeding 20 hours

Activation of Crude Phage Lysate

Freshly prepared phage lysates are known to increase in titer upon standing The phenomenon is thought to be due to inhibiting products of host cell origin being split off from the virus particles The inhibited viruses can be activated by dilution in distilled water and by incubation with diluted anti phage serum (*Sagik* 1954, *Cann & Clark* 1954) No activation by normal serum has been found (*Sagik* 1954)

The effect of normal serum on native T 2 lysate was reinvestigated in the present study as to the significance an activating phenomenon would have on the interpretation of the initial lag phase appearing at inactivation of phages by normal serum

Firstly, a comparison between a fresh and a three month old phage lysate as to the inactivation rate was performed As seen in Fig 9 a definite difference in reaction velocity was found, the difference increasing with dilution of serum

The seemingly slower inactivation of the freshly prepared lysate thus pointed to an activation effect by diluted serum A material of inactivation serum concentration tests on several hundred sera over a period of one year was employed to investigate in a more direct manner if activation occurred In these tests, serum was diluted in 1 5 fold steps and incubated with phages for 2 hours at 37° C

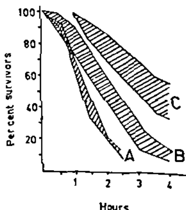


Fig 9
Time surv r curves of fresh and old T2 lysate at various concentrations of normal serum

Final serum diluti n A 1 25 B 1 38 C 1 57 Shad ...

Rea ...

... ure

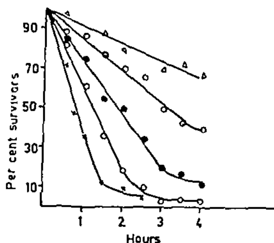


Fig 10

Time-survivor curves of T2 at various concentrations of normal serum presuming activation of original inhibited phages

Analysis of experiment illustrated in Fig 6 Final serum dil from left to right 1 17, 1 25, 1 38, 1 57, 1 85

The plaque counts at the serum dilution next under that giving less than one per cent inactivation were selected Forty-four counts fulfilling these requirements were found representing 15 different sera and 3 different T 2 phage preparations

Except for two sera the plaque counts of the phage-serum dilution mixture were higher than the control counts of the phage-broth mixture The average deviation of all counts from controls were + 9 7 per cent

In experiments to study the reactivation of phages inactivated by normal serum, treatment with proteolytic enzymes increased the titer of the native lysates with on an average 12 5 per cent as will be reported in a following paper

Presuming that the initial number of phage particles in the experiment illustrated in Fig 6 actually was 12 5 per cent higher than revealed by the control platings, the inactivation would have proceeded linearly from the very beginning as shown in Fig 10

DISCUSSION

Kinetic aspects In comparison with the exponential course of the neutralization of T 2 by immune antibodies, the inactivation of T 2 by normal serum was found to be a slower reaction proceeding arithmetically with time As seen in the specific neutralization reaction, the inactivation process brought about by normal serum deviated from the linear course at low and high degrees of inactivation However, a distinct initial lag period increasing with dilution of serum appeared even at high serum concentrations The lengths of the lag periods were also

considerable Thus phage activity decreased less than 5 per cent in one hour in a lot of normal serum of average inactivation capacity diluted 1 57 (Fig 6) For comparison *Cann & Clark* (1954) using an immune serum diluted 1 14150 found a lag period of 15 minutes

In contrast to earlier experience (*Sagik* 1954) normal serum has been found to activate the inhibited virus particles of crude phage lysates Similar to the findings at immune neutralization of T 2 (*Cann & Clark* 1954) the initial lag phase appearing at inactivation by normal serum is probably due to this activating effect

As mentioned after the phase of constant inactivation rate the reaction proceeded at a continuously decreasing velocity leaving a fraction of survivors with normal serum as well as with immune serum It can be concluded from the experiments reported that at higher concentrations of normal serum the magnitude of this persistent fraction was practically independent of the serum dilution At limiting dilutions the size of the fraction was found to be related to the concentration of serum As with immune serum the slope of the linear phase of inactivation seemed to be proportional to the concentration of normal serum At limiting dilutions of normal serum however the rate was slower than expected corresponding to the logarithmic relationship demonstrated between time and inactivation at low serum concentrations (Fig 8)

Quantitative aspects Compared to anti phage serum the inactivating capacity of normal serum can be exhausted within a narrow range of dilution As is evident from the experiment depicted in Fig 8 the dilution 1 100 of a serum of average strength has modified the rate so that 50 per cent inactivation was first reached after seven hours at 37° C

As to the interpretation of the sigmoid inactivation serum concentration curve the deviation from linearity at low concentrations can obviously be ascribed to the lag phase and activating effect just discussed

The midpiece of the curve is linear irrespective of linear or semilogarithmic plot The semilogarithmic plot was chosen for the practical reason of increasing the length of the straight part This, of course, will increase the accuracy of the experiment as the slope may be determined by more data

The cause of the second curvature is still obscure It is not due to dilution factors (*Kallings* to be published), nor to transferable properties of the phage particles Th[illegible]
be the appearance of inhibitors [illegible]
to be overcome [illegible]
trations of ser[illegible] [illegible]ften seen at in
creasing high[illegible] suggestion

The inactivation serum concentration curve thus seems to reflect the effect of at least three competitive activities activation of phages inhibited by products of host cell origin inactivation of phages and inhibition of phage inactivation

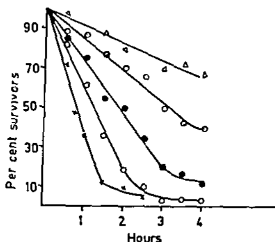


Fig 10

Time survivor curves of T2 at various concentrations of normal serum presuming activation of original inhibited phages

Analysis of experiment illustrated in Fig 6 Final serum dil from left to right 1·17, 1 25, 1·38 1 57, 1·85

The plaque counts at the serum dilution next under that giving less than one per cent inactivation were selected Forty-four counts fulfilling these requirements were found representing 15 different sera and 3 different T 2 phage preparations

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TUBERCULOSIS IMMUNIZATION RESEARCH CENTRE
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INHIBITION OF TRANSPLANTS OF SPONTANEOUS ISOLOGOUS TUMORS IN MOUSE RADIATION CHIMAERAS

By

J. V. SPÄRCK

Received 3.xii.60

As an organism in which the reticulo-endothelial system after its destruction by lethal irradiation has been replaced by a graft of cells from a healthy donor, the radiation chimaera provides a useful means of investigation into certain aspects of the mechanism of immunity and resistance. By using donors of different genetical equipment, or donor material from different developmental stages, it should be possible to gain knowledge about the significance of these variables with respect to the capacity of the organism to react to various antigenic or infectious agents. Furthermore, the radiation chimaera gives a special opportunity of studying long term effects of otherwise lethal irradiation, for instance on immunological competence and resistance.

Studies on tumor transplantation and skin grafting have revealed various modifications of the immune response of the mouse radiation chimaera. *Main & Prehn* (1955) demonstrated than when inbred mice are restored with F_1 bone marrow they become susceptible to skin grafts from both parental strains as well as the hybrid. This was confirmed by *Trentin* (1956) and *Barnes & Loutit* (1959) who further showed that skin grafts from both host strain, donor strain and the F_1 hybrid would also be accepted in the homologous chimaera. Skin graft experiments therefore indicate that the immunological responses of the chimaera are the sums of responses which might be expected from the host and donor components. Similarly, work on transplantation of tumors has revealed that the immune mechanism of the homologous chimaera is not controlled by the host alone, but is influenced by the donor tissue. Thus *Barnes, Ford, Ilbery, Koller & Loutit (1957)* and *Ilbery, Koller & Loutit* (1958) showed that bone marrow and lymph gland donor material used to protect irradiated mice of a different strain can confer on the latter the susceptibilities which characterize the donor. Even after

This work was carried out with the technical assistance of Miss *Else Nielsen* and Miss *Birgitte Engelbrecht Kristensen*. The author is also much indebted to Dr. S. *B[illegible]len* and to Dr. *M. Simonsen* for inspiring discussions and advice. Thanks are further due to the *Radiophysical Laboratory*, the *Radium Centre, Copenhagen*, for the use of X-ray facilities and to Miss *K. B. Storm* for her help in performing the irradiations.

Are the last-mentioned inhibitors ordinarily present in normal serum or are they formed in the course of the inactivation reaction?

Both alternatives seem in fact to be possible. The addition of heat inactivated or zymosan-treated normal serum to a phage-normal serum reaction mixture exhibits a marked inhibiting effect (*Kallings*, to be published). The appearance of inhibitors during the progress of inactivation is supported by the course of inactivation at different temperatures (*Kallings* 1961).

SUMMARY

According to the observations reported, the kinetics of inactivation of T 2 by normal serum seem to have many similarities to neutralization by immune serum. However, an important difference is constituted by the exponential decrease of T 2 particles due to immune antibodies as opposed to a linear decrease due to normal serum.

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the closed colony This line shows a hight spontane us incidence of carcinomas in the female, it is assumed that it possesses the H 2 allele H 2K since a closely related Danish C_3H subline was found to belong to the H 2K phenotype and since Amos (1958) has found a considerable number of C_3H sublines to be H 2K AKA

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exper ments this dose was f und t le the 30 day LD 99 dose for C_3H mice in this laboratory In the later experiments the X ray dose was increased to 750 r For sublethal irradiation the dose was 530 r or 550 r which is about LD 15/30 for our C_3H strain The accumulated dosage was measured just above the wooden cylinder w th a Victoreen Integron IV dos meter

Pro luction of Chimaeras

Cell suspensions of hemat poietic tissues for irradiation therapy were prepare l e ther from fetal l ver or adult spleens or b ne marrow The livers were collected under sterile conditi ns from 17 18 days ld fetuses of C_3H mice or AKA mice A

ac d methylviolet s luti n

C_3H mice 2 3 months of age were used as recipients of fetal liver or adult spleen or bone marrow 1 4 h urs after irradiation with 700 r or 750 r they were injected in the ta l with 0.5 ml f a cell suspension c ntaining al out 4 × 10^7 nucleated cells per ml The mice were thereafter kept singly in jars

TABLE 1

I ro luction of Ra liation-Ch maeras

Tre itment 00 or 750 r followe l by 2 × 10 Hematopoietic Cells i/o

Combination: Recipient	Combination: Donor	Donor-material	Number treated	Dead within days	Dead within 8 20 days	Survivors at 30 days	Per cent survivors: Of all	Per cent survivors: Excluding early deaths
C_3H	C_3H	embryonic liver	215	5	30	180	83 7	85 7
C_3H	C_3H	1 8 months spleen	105	8	30	67	63 8	69 1
C_3H	AKA	embryonic liver	391	16	36	339	86 7	90 4
C_3H	AKA	adult bone marrow	25	3	7	15	60 0	72 0

several months such homologous chimaeras will accept tumors specific for the host as well as tumors specific for the donor strains.

The experiments quoted have also demonstrated that time elapsed since establishment of the chimmaeric state is of importance for the immunological response of the chimaera upon transplantation. It has been found (*Barnes et al.* 1957, *Ilbery et al.* 1958) that for some weeks after treatment the immunological mechanism of the chimaera is non-specifically inactivated in the sense that they are unresponsive to and are killed by implants of homologous, heterologous, and isologous tumors. This neutral state seems to be followed within less than three months by a recovery of the immunological mechanism, the characteristics of which, however, may depend on the genetic relationship between the host and donor. Using skin homografting *Koller & Doak* (1959) found the immunological response of the homologous chimaera shortly after treatment to be of the donor-type, while the characteristics of the late response were more variable, being either donor-type, host-type or ill-defined.

The work of *Koller & Doak* (1959) showed that in case of the isologous radiation chimaera the immunological competence with respect to rejection of tumor homografts is fully restored about 25 days after treatment. It was established that this recovery is due to the activity of the donor material, but it was also shown, that the process of immunological recovery is delayed in isologous chimaeras compared with mice which have only been exposed to a sublethal irradiation dose. Recent work by *Bridges, Loutit & Micklem* (1960) shows that with respect to skin graft rejection the isologous mouse radiation chimaera is characterized by a lasting immunological depression.

Previous work on tumor transplantation in radiation chimaeras has mainly been concerned with the response to homologous tumors or to isologous tumors which had already been transplanted through a great number of passages. Very little attention has been paid to the reaction of radiation chimaeras to transplants of tumors of recent spontaneous origin in the recipient strain. The present work was therefore initiated with the intention of throwing light upon the effect of X-irradiation and subsequent implantation of hematopoietic cells upon the growth of spontaneous isologous tumor grafts. To this end the growth rate of spontaneous C_3H-mouse carcinomas transplanted to normal C_3H-mice was compared with the growth obtained in the same kind of mice previously lethally irradiated and protected by cell-treatment, as well as in C_3H-mice surviving sublethal irradiation without cell-treatment.

MATERIALS AND METHODS

Mouse Strains

The mice used were of the inbred strains C_3H and AKA. C_3H mice were obtained in 1954 from the Institute of Pathology, University of Aarhus, where they had been maintained for several years by brother and sister breeding, but after the transfer to the State Serum Institute they have been maintained by random mating within

the closed colony This line shows a hight spontaneous incidence of carcinomas in the female it is assumed that it possesses the H 2 allele H 2K since a closely related Danish C_3H subline was found to belong to the H 2K phenotype and since Amos (1958) has found a considerable number of C_3H sublines to be H 2K AKA mice were also supplied by the same institute in Aarhus and have been continuously propagated by brother sister matings This AKA line which is a Danish subline of the AKR strain has never shown any spontaneous carcinoma but has a high incidence of leukemia this strain also carries the H 2K allele of the H 2 locus

The mice were kept on a diet of grain and water ad libitum

Irradiation

Irradiation was carried out at the Radiophysical Laboratory the Radium Centre in Copenhagen Groups of 10 mice were irradiated at a time They were placed separately in holes in a cylindrical wooden block which was constantly rotated during irradiation The physical constants of the X radiation were 175 Kv 8 mA 0 5 mm Cu filtration The target distance was 51 5 cm and the dose rate was 40 r per minute The X ray dose used for chimaera production was 700 r in the first experiments this dose was found to be the 30 day LD 99 dose for C_3H mice in this laboratory In the later experiments the X ray dose was increased to 750 r For sublethal irradiation the dose was 530 r or 550 r which is about LD 15 30 for our C_3H strain The accumulated dosage was measured just above the wooden cylinder with a Victoreen Integron IV dosimeter

Production of Chimaeras

Cell suspensions of hematopoietic tissues for irradiation therapy were prepared

[illegible]

[illegible] the further washing the cells were resuspended in Gey s solution

For the preparation of bone marrow suspensions femurs were obtained from adult mice the shafts split open and the marrow scraped out under sterile conditions minced and suspended by the same method as the spleen and liver tissues

Preparation of cell suspensions was carried out at room temperature A haemo [illegible]

[illegible]

TABLE 1

Production of Radiation Chimaeras
Treatment 700 or 750 r followed by 2 × 10 Hematopoietic Cells i/t

Combination		Donor material	Number treated	Died within 7 days	Died within 8 29 days	Survivors at 30 days	Per cent survivors	
Recipient	Donor						Of all	Excluding early deaths
C_3H	C_3H	embryonic liver	215	5	30	180	83 7	85 7
C_3H	C_3H	1 8 months spleen	105	8	30	67	63 8	69 1
C_3H	AKA	embryonic liver	391	16	36	339	86 7	90 4
C_3H	AKA	adult bone marrow	25	3	7	15	60 0	72 0

For the experiments reported here more than 900 C3H mice received X rays and subsequent treatment with either isologous or homologous cells. Table 1 represents the results in terms of the number of surviving mice 30 days after treating 736 irradiated recipients with the two kinds of fetal liver or adult C3H spleen.

Tumor Transplantation

The carcinoma is cut out and removed sterilely from its site under the skin. It is made into a cell suspension in the same way as described above for liver and spleen. The cell number is estimated by means of a haemocytometer. Immediately after its preparation the suspension is divided into equal volumes each being injected subcutaneously into the flank of a recipient, the usual inoculum was 0.1 ml containing 10^{6}–10^{7} cells. The development of a solid tumor in the different recipients was observed by two weekly inspections. When transplanted subcutaneously such tumors grow only locally and do not metastasize, normally they become palpable about two to four weeks after transplantation. A preliminary and approximate estimate of the tumor growth is obtained by registering the two main diameters at each inspection. However, the quantitative method which was preferred consisted in sacrificing all the recipients of an experiment on a certain day, normally 5–6 weeks after transplantation, collecting all the tumor tissue and determining its weight for each recipient of the group and calculating the average tumor weight.

The spontaneous C3H carcinomas used for transplantation in the present investigation normally grew in all isologous recipients of both sexes, and never regressed spontaneously, but they were rejected by AKA-recipients.

EXPERIMENTAL RESULTS

Growth of Spantenous C3H-Tumors Transplanted to Lethally Irradiated C3H-Mice Treated with Embryonic Liver Cells

It was decided to limit this investigation to spontaneous tumors isologous to the recipient strain of the chimaera and not to extend the experiments beyond the fifth transplantation passage. Spontaneous mammacarcinomas from C3H females were grafted into different groups of recipients of the same age, namely normal C3H, normal AKA, isologous chimaeras C3H/C3H, and homologous chimaeras C3H/AKA. The results shown in Table 2 are given in terms of average tumor weights obtained after the transplantation of five different spontaneous C3H carcinomas in the first passages comprising in all 409 recipients.

It is seen that there is a strong tendency towards an inhibition of the growth of all the five tumors in the radiation chimaeras as compared to the normal C3H hosts. This was the case regardless of whether the time lapse between the treatment and tumor transplantation was one month or up to seven to eight monts. Each group consisted of equal numbers of male and female recipients and it was found that for none of the tumors used was there any dependence on sex either in the first or in the later transplantation passages.

It should be pointed out that it was found in many but not all experiments that the radiation chimaeras had a somewhat lower body weight than the normal controls, the difference being up to about 10 per cent. As indicated in Table 2 the tumor weights of the normal C3H recipients were often 2–3 times higher than the weights of tumors in chimaeric hosts. These considerable differences can hardly be explained

TABLE 2

Inhibition of Tumor Growth in Irradiated Mice Protected with Fetal Liver Cells.

Spontaneous C3H tumors	Passage no.	of chimeric ages (days)	Number of days tumor developed	Recipients: Normal C3H		C3H AkA		C3H C3H		Normal AkA	
				Number	Average tumor wt grams	Number	Average tumor wt grams	Number	Average tumor wt grams	Number	Average tumor wt grams
480	1	94	40	7	++	7	0		–	7	0
480	2	78	40	8	++	3	+		–	8	0
480	3	57	47	8	2.12 ± 0.99	8	0.63 ± 0.42	–	–	4	0
480	4	87	46	7	3.77 ± 1.53	6	3.04 ± 0.28		–	4	0
480	5	57	47	9	4.03 ± 1.29	5	2.28 + 1.25	4	2.53 ± 0.56	4	0
501	1	55	39	14	4.23 ± 0.46	15	1.53 ± 0.30		–	16	0
501	2	60	49	1	5.77	7	2.45 ± 0.55	8	1.58 ± 0.59	8	0
501	3	29	35	9	2.32 ± 1.09	10	1.01 ± 0.51	10	1.21 ± 0.45	6	0
501	3	29	34	9	3.34 ± 0.65	10	1.54 ± 0.31	5	1.70 ± 0.57	6	0
513	1	106	53	7	0.55 ± 0.58	10	0.62 ± 0.52	–	–	10	0
513	2	159	45	6	0.60 ± 0.48	6	0.90 ± 0.28	–	–	–	–
513	3	169	35	6	3.83 ± 0.94	6	1.80 ± 1.08	–	–	5	0
513	4	204	28	6	3.57 ± 0.99	5	0.59 ± 0.43	–	–	5	0
513	5	231	32	6	6.93 ± 0.72	6	2.04 ± 0.62	–	–	6	0
560	1	73	29	14	2.13 ± 0.48	12	0.91 ± 0.22	14	1.24 ± 0.23	4	0
561	1	74	50	14	1.55 ± 0.50	10	0.16 ± 0.32	14	0.47 ± 0.24	4	0

The figures presented are the average tumor weights per group ± twice the standard error

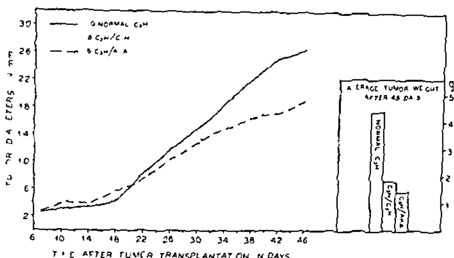


Fig 1

Growth of a C_3H tumor (tumor 630, 1st passage) in normal recipients and in radiation chimaeras treated 22 days before tumor challenge with 750 r X-radiation followed by injection of fetal liver cells *Tumor development is expressed in average diameters* at different times after transplantation The two main diameters of each tumor were measured twice a week with calipers and the average diameter of the group calculated To the right are shown diagrammatically the average tumor weights of the three groups at the termination of the experiment

by the insignificant differences in body weight Furthermore, there was no correlation between the body and the tumor weights obtained in the different experiments, *i e* big tumor weight differences between groups did not mean big body weight differences

It is also apparant that the "chimaeric age", *i e* the time elapsed since treatment, is of no obvious importance for the tumor inhibition In the experiments described here the "chimaeric age" at the time of tumor transplantation varies from 29–231 days without any corresponding variation in the tumor inhibition being observed

A further illustration of the main finding is given in Fig 1, which shows the results of a typical experiment as expressed in the different increases of average tumor diameters with time It appears that the growth curves in this respect are similar in the different groups approximating straight lines

Growth of Spontaneous C_3H-Tumors Transplanted to Lethally Irradiated C_3H-Mice Treated with Adult Cells

The observation of a reduced susceptibility of the radiation chimaeras for tumors which had arisen spontaneously in the recipient strain raises the question whether this protective effect is due to the implanted donor cells or to irradiation If the colonising donor cells possess a capacity which is lacking in the recipients there could theoretically be two possible answers to the question Firstly, the tumor inhibiting

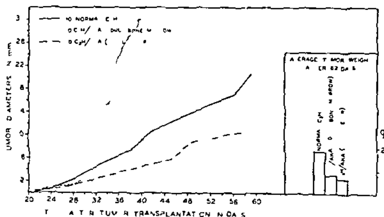


Fig 2
Development of a C3H tumor (tum r 586 2nd passage) in normal recipients and in rad ation chimaeras treated 34 days before tumor challenge with 750 r X radiation followed by injection f either h mologous adult bone marrow or homologous fetal liver F r further explanation see Fig 1

effect of the donor cells could be due to the fact that they are derived form a genetically different strain which is resistant to tumors of the type in question This assoumption seems however, to be ruled out by the data presented in the preceeding section which show that the tumor inhibition is of the same order of magnitude in both the isologous and the homologous chimaeras Secondly the phenomenon could be due to the fact that the donor cells are from an organism at a different developmental stage characterized by a better capacity to regulate neoplastic growth than the recipient

Since both kinds of chimaeras described above, isologous and homologous are also age chimaeras" namely 2 3 months old recipients and fetal donors the possibility therefore remains that the embryonic state of the tissue was responsible for the transferred protective capacity The role which the source and age of the donor material plays for the changed susceptibility of the radiation chimaera to isologous spontaneous tumors was therefore studied As yet only a few results with adult spleen and bone marrow have been collected They are presented in Table 3 summarizing results with 126 recipients, while Fig 2 illustrates a single experiment

These experiments indicate that irrespective of whether the restorative cells are from spleen or bone marrow or they are of isologous or homologous origin the resulting radiation chimaeras are in all cases characterized by an inhibition of spontaneous isologous tumor grafts which is of the same order of magnitude On the whole the results with the radiation chimaera suggest than when C3H mice which are of the same age are irradiated lethally and subjected to a postirradiation treatment with hematopoitic cells they acquire a characteristic di

TABLE 3

Inhibition of Tumor Growth in Irradiated Mice Protected with Different Adult or Fetal Cells

Spontaneous C_3H tumor no	Passage no	Donor material	*Chimeric age* (days)	Number of days tumor developed	Recipients							
					Normal C_3H		C_3H AKA		C_3H C_3H		Normal AKA	
					Number	Average tumor wt grams	Number	Average tumor wt grams	Number	Average tumor wt grams	Number	Average tumor wt grams
554	4	adult spleen	36	25	10	*6.13* ± 0.83		–	21	*1.98* ± 0.30	10	0
586	1	adult spleen	40	49	15	2.84 ± 0.55			10	*0.28* ± 0.18	10	0
586	2	adult bone marrow	34	62	10	*1.83* + 1.42	10	*0.79* ± 0.79		–	10	0
586	2	fetal liver	34	62	10	*1.83* ± 1.42	10	*0.64* ± 0.68	–		10	0

The figures presented in the table are the average tumor weights per group in grams ± twice the standard error

minished susceptibility to transplants of spontaneous isologous carcinomas. This diminished susceptibility seems to be independent of a considerable variation in the donor material with respect to stage, organ and genotype.

Growth of Spontaneous C_3H Tumor Transplants in C_3H Mice Sublethally Irradiated without Subsequent Cell Treatment

In connection with the studies described above some other experiments were carried out in order to ascertain whether the whole body irradiation as such is sufficient in bringing about an inhibition of the growth of spontaneous isologous tumors. For example a number of two months old C_3H mice were irradiated with 530 r or 550 r X-ray, a dose which is about LD 15/30. The survivors were thereafter challenged together with normal C_3H mice of the same age with different spontaneous C_3H carcinomas at different times after irradiation. The result with respect to tumor growth is presented in Table 4.

TABLE 4

Tumor Growth in Normal and 530r Irradiated C_3H Mice

Tumor	Pass no.	Recipients	Interval between irradiation and tumor inoculation (days)	Average tumor weight grams	Days allowed for the tumor to develop
630	1	8 irrad. C_3H	22	2.17 ± 0.36	48
"		10 norm. C_3H		4.39 ± 0.91	"
554	1	10 irrad. C_3H	32	0.75 ± 0.15	28
"	"	10 norm. C_3H		0.85 ± 0.27	
554	2	10 irrad. C_3H	60	4.13 ± 0.46	35
"		10 norm. C_3H		7.41 ± 0.81	
560	2	10 irrad. C_3H	32	0.39 ± 0.24	67
"		10 norm. C_3H		1.28 ± 0.44	"
560	3	10 irrad. C_3H	60	1.44 ± 0.33	37
"		10 norm. C_3H		2.69 ± 0.38	"

The figures presented are the average tumor weights per group ± twice the standard error.

It appears that the tumors grow considerably less in the irradiated recipients, a condition which does not seem to be altered even over a relatively long period, since the inhibition is of the same order of magnitude 22 and 60 days after the treatment.

DISCUSSION

Two basic findings [illegible] radiation chimeras h[illegible] hat transplants of spontan[illegible] [illegible] tumors isologous to the recipient irrespective of the origin of the restorative donor cells, and (2)

that mice surviving a sublethal X-irradiation have acquired a similar resistance to implants of isologous tumors of recent spontaneous origin

The observation of an inhibited growth of spontaneous tumors upon transplantation to irradiation chimaeras of both isologous and homologous compositions appears rather surprising as other workers, on the contrary, report a reduced immunological reactivity of radiation chimaeras against tumor transplants, a state which is claimed to persist even after the immunological neutral post-irradiation period is over In other words, tumors compatible with either the recipient or the donor component of the chimaera have been found by others to grow better upon transplantation to chimaeric hosts than they do in normal control animals

With regard to this apparent discrepancy between the findings quoted and the present experiments it must be borne in mind that the former observations were based on transplantations of either tumors foreign to the recipient organism or tumors originating in the recipient strain but already transplanted through so many passages (in the ascites form) that they may well be antigenically changed This latter type of transplantation does therefore not necessarily represent isotransplantation in the strict sense of the word In our experiments on the other hand we have been dealing with a principally different situation, namely the transplantation of tumors immediately after they have arisen spontaneously in the strain which enters the chimaera as recipient In the case of experiments such as those of *Ilbery et al* (1958) or *Koller & Doak* (1959) we seem to be concerned with the modified reaction of the immune mechanism to transplant antigenically foreign to the recipient, while the type of isograft reaction with which we are dealing in our experiments may well be based on a different mechanism which is influenced in a different manner by X-irradiation

The possibility of modifying the immunological specificities of the irradiated recipient by resuscitation with different kinds of cells led to the suggestion some years ago (*Barnes, Corp, Loutit & Neal* 1956) that tumor cells surviving the irradiation could be eliminated by antibodies produced by implanted homologous hematopoietic tissues *DeVries & Vos* (1958) observed that when a C57Bl-lymphosarcoma was transplanted to C57Bl-mice and the recipients were lethally irradiated 4 6 days later, the injection of homologous or heterologous lymph gland cells together with bone marrow resulted in inhibition of tumor growth Isologous lymph gland cells in the same quantity did not have such an effect The inhibition of tumor growth in case of treatment with homologous cells is possibly just an effect of a general graft versus host reaction, since the recipients died shortly after from "secondary disease"

However, these findings can not be compared with the results reported here, partly because the lymphosarcoma used by these authors was already transplanted through very many passages, and partly because the treatment was applied after inoculation of the tumor

From the present investigation it seems justified to conclude that the inhibited growth in radiation chimaeras of spontaneous tumors isologous to the recipient is mainly caused by X irradiation as such, since application of a considerable diversity of donor material result in tumor inhibition of the same order of magnitude and since a sublethal dose of X rays without cell treatment also causes a long lasting resistance to this type of isologous tumor grafts

With regard to the effect of a previous whole body radiation on tumor growth it was shown by *Murphy*, as early as 1914, that sublethal irradiation of mice and rats reduces the resistance to homologous and heterologous tumor grafts by which the foreign tumors are enabled to grow in these animals It has also been shown that after a few weeks the resistance is re established (see *Clemmesen* 1937, *Koller & Doak* 1959) This course of events seems to correspond well to the transient destruction of the antibody forming mechanism which has been found following a moderate total body irradiation (see *Taliaferro* 1957) That radiation chimaeras are found to be immunologically inactivated in certain respects for some weeks after treatment seems likewise to reflect this X ray effect

The experiments which are presented here demonstrate that when a *spontaneous* tumor *isologous* to the recipient is implanted the effect of a previous whole body irradiation is quite opposite to the effect known from cases of homo and heterotransplantation the growth of the isologous tumor graft is strongly reduced Furthermore, it is shown that this insusceptibility persists for up to several months after irradiation This observation seems to be of interest in connection with the problem of whether the radiation effect on tumors depends mainly on a direct effect on the tumor tissue or whether on the other hand a systemic response to irradiation plays the major role in the inhibition phenomenon

So far as is known to the author no other reports are available on the influence of previous whole body irradiation on the growth of transplants of isologous spontaneous carcinomas in mice However *Hollcroft, Lorenz & Hunstiger* (1951) have treated inbred mice 10 days *after* subcutaneous inoculation of an isologous but long transplanted lymphosarcoma with different doses of whole body irradiation and obtained a transient regression of the tumor This work demonstrated clearly that an indir[illegible] systemic reaction is involved in the tumor inhibition If, for inst[illegible] tumor is irradiated locally with 1300 r only a slight effect is obt[illegible] When on the other hand 1300 r by means of a shielding devic[illegible]tributed so that the tumor receives 1000 r locally, while the rest of [illegible]dy receives 300 r a highly increased regression effect is obtain[illegible]

Th[illegible] rvations reported here differ from those just mentioned above [illegible]ing with the inhibition of tumor growth in recipients which were [illegible]diated up to several months previously It is not possible at

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present to state anything conclusive about the mechanism responsible for this phenomenon but further experiments are being carried out to elucidate the nature of the modification underlying the lowered susceptibility of the host

SUMMARY

The effect of lethal whole-body irradiation of C_3H-mice, followed by restoration with hematopoietic cells, on the growth of subsequently implanted primary or early-passage spontaneous C_3H-carcinomas has been investigated

In contrast to what has been established previously in the case of homotransplantation, against which radiation chimaeras have been shown to have a reduced immunological reactivity, it was found that irradiated C_3H-mice protected with hematopoietic cells acquired an increased resistance to the outgrowth of grafts of spontaneous C_3H-tumors This acquired insusceptibility to isologous tumors was found to be of the same degree irrespective of whether the chimaera was of isologous or homologous composition, or whether the restorative donor-material was embryonic liver, adult spleen, or bone marrow The acquired tumor-insusceptibility was present to the same extent irrespective of whether tumor-challenge was performed 3 weeks or up to 8 months after treatment

Experiments on sublethal irradiation (550 r) of C_3H-mice and subsequent challenge of the survivors with isologous spontaneous carcinomas demonstrated that irradiation alone could produce a corresponding tumor inhibiting effect Also this is contrary to the well established transient decrease in resistance to homo- and heterografts following a previous whole-body irradiation The reported insusceptibility to tumors of isologous origin following a sublethal irradiation was found to persist for at least a few months

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BRIEF REPORT

SENSITIVITY DETERMINATION OF TUBERCLE BACILLI TO CYCLOSERINE BY THE SLIDE CULTURE TECHNIQUE

By *Gunnar Haukenes*

In vitro investigations have shown that most strains of human tubercle bacilli a susceptible to 10 to 15 µg cycloserine per ml (*Cummins et al* 1955 *Steenken Wolinsky* 1956 *Weisert* 1958)

Cycloserine is relatively stable to alkali but is readily degraded in acid (*Goldbe* 1959) *Cummins et al* (1955) found a loss of the drug of 40 per cent in a liqui medium after 7 days at 37° C *Weisert* (1958) investigated the stability of cycloseri during storage at various temperatures He found a reduction of 21 per cent aft 10 days at 37° C Both figures refer to a concentration of cycloserine of 10 µg/ml i a slightly acid medium These data cannot without reservations be applied to othe methods for sensitivity determination and a short report on the results obtained b the slide culture technique (*Oeding* 1951) may be of interest

Smears of strain H37Rv and an INH resistant strain were cultivated in bloo water prepared by hemolyzing citrated whole blood with three volumes of distille water The pH of the medium is about 7 45 Concentrated solutions of cycloserin in a phosphate buffer of pH 7 6 were added to the blood water to the final concen trations of 1 2 5 and 10 µg/ml Two controls without cycloserine were included Th

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ance with those obtained by *Steenken & Wolinsky* (1956) in a liquid medium of pH 7 8 The stability of cycloserine under the conditions employed was examined Since solutions of cycloserine were never stored the main problem was whether the activity of the drug would decrease during the growth period Solutions in blood water of the above mentioned concentrations of cycloserine were stored for 7 days at 37° C before being used for cultivation Comparison was made with freshly pre pared solutions of the drug in the same batch of blood water The test strain in this experiment was H37Rv The experiment was carried out in duplicate with a different batch of blood water

No deterioration of the activity was detected during the growth period The colori metric method for determination of cycloserine was not applicable to blood water solutions Ordinary bacteriological methods cannot display a minor loss of anti biotic activity but in this case the loss if any must have been negligable since the size of the colonies in the concentration 5 µg/ml was unaltered It is our experience that the microscopical examination of the slides enables a fine and reliable grading of growth inhibition

The slide culture technique seems to be well suited for sensitivity determination of tubercle bacilli against cycloserine The mode of action of cycloserine was pri

were reduced in size but not in number The

xplain the stability of the drug in our experi

following concentrations of cycloserine are

owth in 25 µg/ml indicates incipient develop

ment of resistance 1959) but the relation to clinical results is still not clear

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